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PRECLINICAL AND CLINICAL ISSUES IN ALZHEIMER'S DISEASE DRUG RESEARCH AND DEVELOPMENT

Topic Editors
Cesare Mancuso and Silvana Gaetani

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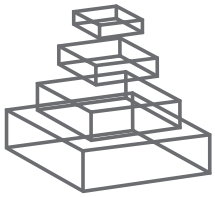

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PRECLINICAL AND CLINICAL ISSUES IN ALZHEIMER'S DISEASE DRUG RESEARCH AND DEVELOPMENT

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Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by progressive cognitive dysfunction and memory loss, inability to perform the activities of daily living and mood disorders. According to the so-called "amyloid cascade hypothesis", amyloid-β-peptide (Aβ), produced by beta- and gamma- secretase-mediated cleavages of the amyloid precursor protein (APP), plays a pivotal role in the pathogenesis of AD. Aβ was also shown to contribute to AD pathology by stimulating the hyperphosphorylation of tau which is responsible for the formation of neurofibrillary tangles. However, the "amyloid cascade hypothesis" was challenged by other theories which lend support to the idea that Aβ is not causative but can be considered as an "innocent bystander" in AD. Although preclinical research generated impressive lines of evidence about the several intracellular mechanism(s) whose impairment leads to the onset and progression of AD, clinical research aimed at the development of new drugs capable of preventing or delaying the onset of neuronal damage in AD patients has produced limited results. The drugs currently available for the treatment of AD are acetylcholinesterase inhibitors (AChEI) and the NMDA glutamate receptor antagonist memantine. The AChEI increase acetylcholine levels in the synaptic cleft, which are reduced because of the progressive damage of cholinergic neurons in cognitive brain areas (e.g. amygdala, hippocampus, and frontal cortex), whereas memantine is used to prevent/reduce calcium-dependent excitotoxic neuronal cell death. Both classes of drugs have been shown to improve symptoms related to cognitive decline, but their effects are confined largely to patients with mild to moderate AD, in particular during the first year or so of treatment. An alternative to this symptomatic treatments involves the use of drugs that intervene in the pathogenesis of the disease. Recently, monoclonal antibodies against Aβ were proposed as novel agents capable to remove Aβ from the brain thus preventing neuronal damage. The research topic focuses on the preclinical and clinical evidence about the several factors that contribute to the pathogenesis of AD as well as the potential therapeutic role of new classes of drugs still under preclinical or clinical development.

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Preclinical and clinical issues in Alzheimer's disease drug research and development

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Alzheimer's disease (AD) is a chronic, rapidly progressive neurodegenerative disease, characterized by loss of memory and cognitive abilities, inability to carry out regular daily activities and mental disorders. The following numbers provide an idea of the extent of the problem: 36 million people worldwide suffer from dementia, of which 7.7 million new cases each year, and 60–70% of them are diagnosed with AD. Patients with AD deal with numerous problems, one of the most relevant being the lack of drugs that can slow down or hinder disease onset. Indeed, the currently available drugs, namely acetylcholinesterase inhibitors donepezil, rivastigmine, and galantamine, and the NMDA receptor antagonist, memantine, are able to counteract or delay the symptoms related to cognitive decline and nearly entirely in individuals with mild-to-moderate AD. The shortage of drugs available for the treatment of AD is also related to the difficulties faced by many pharmaceutical companies, including some Big Pharma, that despite significant achievements from their hit/leads in preclinical and early stages of clinical research, have not been able in recent years, to launch their products on the market due to ineffective results in terms of efficacy or safety of advanced clinical trials. This problem is at least in part due to investigators' difficulties in identifying the most adequate outcomes in order to plan clinical trials. Indeed, in many cases, attention has been focused on certain plasma or cerebral-spinal fluid (CSF) biomarkers, both for the relative ease of sampling and for the possibility they could offer to monitor the disease progression. However, it is worth emphasizing, that despite clinical trials based on the use of peripheral biomarkers have provided encouraging results on the ability of some drugs to block the production or deposition of β-amyloid (Aß) or even destroy existing senile plaques, key phase III studies, specifically designed to evaluate the improvement of cognitive activity, have shown a reduced ability of these new molecules to enhance memory or the ability to perform regular daily living functions in patients with AD or, in some cases have also resulted in severe side effects. Another limitation of pharmacological research on AD is the scarce ability to enhance collaboration between pharmaceutical industries and public or private non-profit organizations conducting basic research, such as universities. Both these sectors would benefit from the strengthening of this cooperation allowing non-profit institutions to enhance basic research on AD by receiving funds from pharmaceutical companies that, in turn, could benefit from the research of qualified scientists worldwide.

This Research Topic has been designed as a platform to analyze some of the most relevant problems posed by research on AD, suggest and develop new ideas that may improve both preclinical and clinical research on this disease. This Research Topic in which leading experts in the field of AD have contributed, is organized in 5 review articles, 3 original research articles, 1 opinion article, 1 Perspective article and 1 Commentary.

The complexity of the events that determine the onset and progression of AD is emphasized by the many factors that contribute to the pathogenesis of this disease. Recently, what has been pointed out is the pathogenetic role played by posttranslational modifications, such SUMOylation, on proteins involved in the production of Aß (Nisticò et al., 2014). In light of this, it is worth noting the remarkable discovery in Tg2576 transgenic mice, a validated preclinical model for the study of AD, that the imbalance of the phenomena of protein SUMOylation/deSUMOylation occurs in cognitive brain areas, such as the hippocampus and cortex, in the very early stages of the disease. This finding certainly contributes to indicate an alternative target for the development of new drugs for AD (Nisticò et al., 2014). The importance of having predictive preclinical models for the proper study of AD is also pointed out by Davis and Head (2014) who suggest the aged canine as an useful animal species for studies on AD and as an alternative to rodent models. Indeed, it has been shown that elderly beagle dogs have a progressive loss of cognitive functions with age and they show characteristic neuropathological hallmarks of AD (Davis and Head, 2014). This canine model allowed preclinical studies on the role of neuroprotective drugs, such as statins. An interesting link between the pathogenesis of AD and of other, apparently different, diseases, such as glaucoma, has been highlighted in a study by Trovato Salinaro et al. (2014). In fact, both AD and glaucoma have a common pathogenetic role played by free radicals and the ability to activate the adaptive stress response systemically (Trovato Salinaro et al., 2014). As in many cells, the activation of cell stress response is a response mechanism to the damage caused by free radicals and its modulation by nutritional supplements, defined nutraceuticals, is currently considered an adjuvant approach to standard therapy of diseases, including AD (Mecocci et al., 2014). The results by Scuderi et al. (2014) seem to support this theory, showing that

the up-regulation of the sirtuin system, *via* resveratrol, is able to reduce the activation of astrocytes and the production of inflammatory mediators in primary cultures of rat astrocytes treated with Aß(1–42). The causal role of infectious agents in relation to the pathogenesis of AD has been little experimented. As reported by Piacentini et al. (2014), recurrent infection by herpes simplex virus type I (HSV-1) can be considered a risk factor for AD. The mechanism(s) through which HSV-1 could give rise to AD are not completely understood, even if the HSV-1–mediated proteolytic processing of APP, generating also Aβ40 and Aβ42 monomers or small oligomers, is among those which received the greatest consensus (Piacentini et al., 2014).

Among the issues with the highest translational impact on research in the field of AD is the use of "old" drugs such as vitamin D, currently used for years in the treatment of bone loss, above all in the elderly, whose neuroprotective properties in subjects with AD have been recently brought to light (Annweiler et al., 2014). A very updated and comprehensive review article by Cornelius et al. (2014) examines the factors that can be considered as a common denominator of AD and osteoporosis. The dysregulation of the endocannabinoid system, initially based on a number of preclinical evidence, as a viable mechanism to intervene in the pathogenesis of AD is no longer controversial (Aso and Ferrer, 2014). Furthermore, the availability of "new" pharmacological tools to modulate the endocannabinoid system of the brain, one with the preclinical evidence of their effectiveness in terms of a significant reduction of oxidative and metabolic damage load on neurons and objective improvements of behavior in individuals with AD, leads research further toward the study of the endocannabinoid system as a novel therapeutic target in AD (Aso and Ferrer, 2014). An example of how a virtuous collaboration between non-profit institutions, such as universities, and pharmaceutical companies should develop is in the article by Lundkvist et al. (2014). This paper focuses on the activities of "AlzeCure," a non-profit Swedish foundation founded by brilliant researchers with a strong background in R&D from a Big Pharma such as Astra Zeneca. The AlzeCure acts as a catalyst, receiving continuous feedback from biotech companies, and leading academic institutions (e.g., Karolinska Institute) involved in R&D of drugs for AD, collaborating in the preclinical and clinical field, demonstrating that a useful collaboration between non-profit institutions and pharmaceutical companies is actually possible (Lundkvist et al., 2014). A viable alternative to this model of academic-industry cooperation is the one proposed by Beauchet et al. (2014) who, in their Commentary, described the activities of "Biomathics," an emerging scientific research consortium whose task is to promote cooperation among the academic research teams working in the field of aging and longevity. One of the main goals of this Consortium is to foster the creation of large research groups, formed by scientists worldwide, so as to increase the possibility of applying and obtaining research grants to enhance international basic research (Beauchet et al., 2014).

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REFERENCES

Annweiler, C., Karras, S. N., Anagnostis, P., and Beauchet, O. (2014). Vitamin D supplements: a novel therapeutic approach for Alzheimer patients. *Front. Pharmacol.* 5:6. doi: 10.3389/fphar.2r.2014.00006

Aso, E., and Ferrer, I. (2014). Cannabinoids for treatment of Alzheimer's disease: moving toward the clinic. *Front. Pharmacol.* 5:37. doi: 10.3389/fphar.2r.2014.00037

Beauchet, O., Merjagnan, C., and Annweiler, C. (2014). From industrial research to academic discoveries, towards a new concept of partnership: the Biomathics model. *Front. Pharmacol.* 5:166. doi: 10.3389/fphar.2r.2014.00166

Cornelius, C., Koverech, G., Crupi, R., Di Paola, R., Angela, K., Lodato, F., et al. (2014). Osteoporosis and Alzheimer pathology: role of cellular stress response and hormetic redox signaling in aging and bone remodeling. *Front. Pharmacol.* 5:120. doi: 10.3389/fphar.2r.2014.00120

Davis, P. R., and Head, E. (2014). Prevention approaches in a preclinical canine model of Alzheimer's disease: benefits and challenges. *Front. Pharmacol.* 5:47. 10.3389/fphar.2014.00047

Lundkvist, J., Halldin, M. M., Sandin, J., Nordvall, G., Forsell, P., Svensson, S., et al. (2014). The battle of Alzheimer's Disease – –the beginning of the future Unleashing the potential of academic discoveries. *Front. Pharmacol.* 5:102. doi: 10.3389/fphar.2014.00102

Mecocci, P., Tinarelli, C., Schulz, R. J., and Polidori, M. C. (2014). Nutraceuticals in cognitive impairment and Alzheimer's disease. *Front. Pharmacol.* 5:147. doi: 10.3389/fphar.2014.00147

Nisticò, R., Ferraina, C., Marconi, V., Blandini, F., Negri, L., Egebjerg, J., et al. (2014). Age-related changes of protein SUMOylation balance in the AβPP Tg2576 mouse model of Alzheimer's disease. *Front. Pharmacol.* 5:63. doi: 10.3389/fphar.2014.00063

Piacentini, R., De Chiara, G., Li Puma, D. D., Ripoli, C., Marcocci, M. E., Garaci, E., et al. (2014). HSV-1 and Alzheimer's disease: more than a hypothesis. *Front. Pharmacol.* 5:97. doi: 10.3389/fphar.2014.00097

Scuderi, C., Stecca, C., Bronzuoli, M. R., Rotili, D., Valente, S., Mai, A., et al. (2014). Sirtuin modulators control reactive gliosis in an *in vitro* model of Alzheimer's disease. *Front. Pharmacol.* 5:89. doi: 10.3389/fphar.2014.00089

Trovato Salinaro, A., Cornelius, C., Koverech, G., Koverech, A., Scuto, M., Lodato, F., et al. (2014). Cellular stress response, redox status, and vitagenes in glaucoma: a systemic oxidant disorder linked to Alzheimer's disease. *Front. Pharmacol.* 5:129. doi: 10.3389/fphar.2r.2014.00129

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Vitamin D supplements: a novel therapeutic approach for Alzheimer patients

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INTRODUCTION

Alzheimer disease (AD) is the main cause of dementia and loss of functional independence in the elderly (Aisen, 2010; Vellas and Aisen, 2010). AD is a chronic neurodegenerative disease characterized by a progressive decline of cognitive performance with a deleterious impact on social activities. AD is a worldwide big concern because of its adverse consequences and its expanding prevalence and incidence (Aisen, 2010; Vellas and Aisen, 2010). In order to reduce AD impact at an individual level and in terms of health and social costs, the development of efficient therapeutic strategies proves necessary. Regrettably, the development of all current specific medications is unsuccessful in clinical development phases (Birks, 2006; McShane et al., 2006; Anand et al., 2014). However, an interesting fact is that the prevalence of dementia in the general population might still be subject to changes. Indeed, a reduction in the prevalence of dementia is reported among English community-dwellers over the past two decades (Matthews et al., 2013). So, even if some factors, such as the increased life expectancy beyond 80 years, are unavoidable and augment the global prevalence of dementia, others may instead reduce its prevalence. For instance, efficient primary prevention of cardio-vascular disease is suspected to explain the reduced prevalence of dementia in England (Matthews et al., 2013). In the same vein, vitamin D, an "old" molecule primarily known for its effects on the bone, has been singled out in the last decade as an important biological component able to influence the natural history of AD. Here, we wish to highlight the difficulties experienced by the research on curative treatments and, in contrast, the prospects offered by multi-target drugs, especially those based on vitamin D.

TREATMENTS RESEARCH IN ALZHEIMER DISEASE: THE IRONY OF CURATIVE THERAPIES

The only drugs currently available (i.e., cholinesterase inhibitors and memantine) slow down without changing the natural history of AD (Birks, 2006; McShane et al., 2006). Because of their symptomatic action, they are intended only for patients with mild-to-severe AD. Thus, one of the main AD challenges over the coming decade lies in the finding of a curative drug (Vellas and Aisen, 2010). Recent research has focused on "disease-modifying" medications to intervene in the pathogenesis of the disease, able to halt or slow the neurodegenerative process. To date, strategies to develop disease-modifying drugs have mainly targeted amyloid-β peptide (accumulation, aggregation, clearance) and/or the "tubulin associated unit" (TAU protein: phosphorylation and aggregation) by active and passive immunotherapy (Vellas and Aisen, 2010; Anand et al., 2014). Unfortunately candidate drugs, such as bapineuzumab, have failed in phase III clinical trials conducted in mild-to-moderate AD (Anand et al., 2014).

Because the earlier the disease-modifying treatment starts, the greater is the possibility of efficacy, it is important to initiate the treatment before the clinical manifestation of the overt disease. This is one of the reasons of the interest for the early diagnosis of AD. Importantly, this research has identified new morphological as well as functional and biological criteria that, combined with the classical neuropsychological expertise, help make the diagnosis of AD earlier and earlier (Schrötter et al., 2013). Such accuracy of the diagnosis of early-stage AD led in memory clinics to a drastic selection of participants for the curative clinical trials, with consequent substantial recruitment difficulties. The second consequence of this hyperselection was to address only a fraction of the patients followed in memory clinics, predominantly those with the prodromal stage of AD. For instance, the French National Centre for the Management of Trials on Healthcare Products (CeNGEPS), which involves more than ten university memory clinics (UMCs) in France, included in 2009 only 260 participants in clinical trials, although there were potentially 24,000 patients available in these centers. In other words, the curative drugs are addressing only 1.1% of AD patients followed in French UMCs. The question is then what can be offered to all other patients.

TREATMENTS RESEARCH IN ALZHEIMER DISEASE: THE MULTI-TARGET DRUGS

In addition to curative treatments, two other therapeutic approaches should be considered in AD. The first one is the non-drug-based approach, which is a multi-domain intervention designed to stimulate

AD patients (de Sant'Anna and Morat, 2013). The second one is the multi-target drug approach, meaning that the treatment administered to AD patients simultaneously targets several neurodegenerative processes (Cavalli et al., 2008). The latter approach offers the opportunity to combine current standard anti-dementia symptomatic drugs with other neuroprotective agents to build a multi-regimen with multi-target effects. This questioning is central since patients at later stages of AD (i.e., with symptoms) could benefit from this new therapeutics, thus reducing chance inequalities between AD patients. In addition, it could benefit a larger number of patients and could be easily implemented compared with current trials, since it targets diagnosed patients who are already using a symptomatic treatment. Among the candidate molecules is the vitamin D, which has demonstrated in the last decade numerous neurological effects.

VITAMIN D AND ALZHEIMER DISEASE: FROM AN INTRIGUING IDEA TO A THERAPEUTIC OPTION

About one billion people are deficient in vitamin D throughout the world. Older adults are especially affected, in particular those with AD with a prevalence of approximately 70–90% (Holick, 2007; Annweiler et al., 2011).

Besides its classical function of bone metabolism regulation, vitamin D has exhibited multiple biological targets mediated by its nuclear hormone receptor, the Vitamin D Receptor (VDR) (Kalueff and Tuohimaa, 2007; Annweiler et al., 2010). Specific actions on target organs such as the central nervous system (CNS) have been described, providing evidence for a neurosteroid action of vitamin D (Kalueff and Tuohimaa, 2007). In particular, VDRs are present in neurons and glial cells of the hippocampus, cortex and sub-cortex, all regions essential to cognition. The binding of vitamin D on the VDRs triggers neuronal protection against AD degenerative processes, including anti-inflammatory action, antioxidant effect, control of calcium homeostasis by regulating the concentration of intracellular calcium in hippocampal neurons, anti-atrophic effect by regulating neurotrophic agents, attenuation of Aß42 peptide accumulation by stimulating the phagocytosis of Aß peptide together with enhancing brain-to-blood Aß efflux transport at the blood-brain barrier, and the prevention of acetylcholine defect by increasing the activity of choline acetyltransferase (thus the bioavailability of acetylcholine) in the brain (Kalueff and Tuohimaa, 2007; Annweiler et al., 2010; Annweiler and Beauchet, 2011). These experimentally-described neuroprotective properties of vitamin D may help, in the case of normalized vitamin D status, to address against the decline of brain function in AD, especially against cognitive decline (**Figure 1**).

Epidemiology is consistent with this notion, and has repeatedly shown that low serum concentrations of vitamin D are cross-sectionally associated with global cognitive impairment (Annweiler et al., 2009), the people with hypovitaminosis D having more cognitive disorders (Etgen et al., 2012), specifically more executive dysfunctions (Annweiler et al., 2013a). Specifically, it has been reported that people with AD have lower vitamin D status than controls (Annweiler et al., 2013b). They also exhibit increased levels of vitamin D-binding protein (Moon et al., 2013) and lower levels of 25-hydroxyvitamin D in the cerebrospinal fluid (Johansson et al., 2013) compared to controls. Longitudinal prospective studies have enabled to better understand this association and have reported a temporal sequence, with hypovitaminosis D preceding the onset of cognitive decline (Llewellyn et al., 2010).

Recent biomedical literature further suggests that the VDRs may confer genetic risk for AD. Some human variants appear less sensitive to vitamin D and more likely to experience cognitive decline. For instance, AD patients have reduced levels of VDR mRNA (Sutherland et al., 1992). In contrast, it has been reported that the overexpression of VDR may suppress amyloid precursor protein transcription (Wang et al., 2012). A significant association has also been shown between the VDR gene *APA1* polymorphism and the onset of AD, the *Aa* genotype multiplying by 2.3 the risk of AD compared to the *AA* genotype (Gezen-Ak et al., 2007).

It seems thus crucial to maintain high vitamin D levels in the elderly, especially in those with AD. In line with this, high intake of vitamin D (whether from food, supplements, or sun exposure) is associated with better cognitive function in older individuals. Having more than 800UI of vitamin D supplies per day divides the risk of AD by 5 after 7 years of follow-up (Annweiler et al., 2012a). This neuroprotective effect has been confirmed by clinical comparative trials reporting cognitive improvement after vitamin D supplementation whether in general aged population (Prybelski et al., 2008; Annweiler et al., 2012b) or in patients who already have symptoms of AD (Stein et al., 2011). The cognitive benefits of supplementation appear at 4 weeks (Prybelski et al., 2008), and particularly for executive functions and processing speed (Annweiler et al., 2013a).

It has to be noted yet that, although most of older adults have hypovitaminosis D, not all develop AD. It is thus unlikely that hypovitaminosis D alone explains the onset of AD, and that vitamin D supplementation is sufficient to prevent AD by itself. One solution might thus be to combine vitamin D with a symptomatic anti-dementia treatment to build a multi-target drug (Annweiler and Beauchet, 2012). Indeed, if hypovitaminosis D explains in part the pathological process of AD, it may also enhance the effectiveness of standard anti-dementia treatments or account at least partially for the resistance to these treatments. Even speculative, this engages clinicians to replenish vitamin D before starting anti-dementia treatments or to use vitamin D as an adjunct to standard treatments. In line with this, a recent 6-month controlled trial has recently reported that the combination of memantine + vitamin D was superior to memantine alone and vitamin D alone in preventing cognitive decline among AD participants (Annweiler et al., 2012c). In fact, those taking both molecules had a clinically relevant and statistically significant gain of 4 points on the Mini-Mental State Examination. These results were consistent with an *in vitro* experiment, which showed that cortical axons degenerate less after exposure to amyloid-β peptide or glutamate in microfluidic neuronal cultures enriched with memantine plus vitamin D compared to control medium and compared

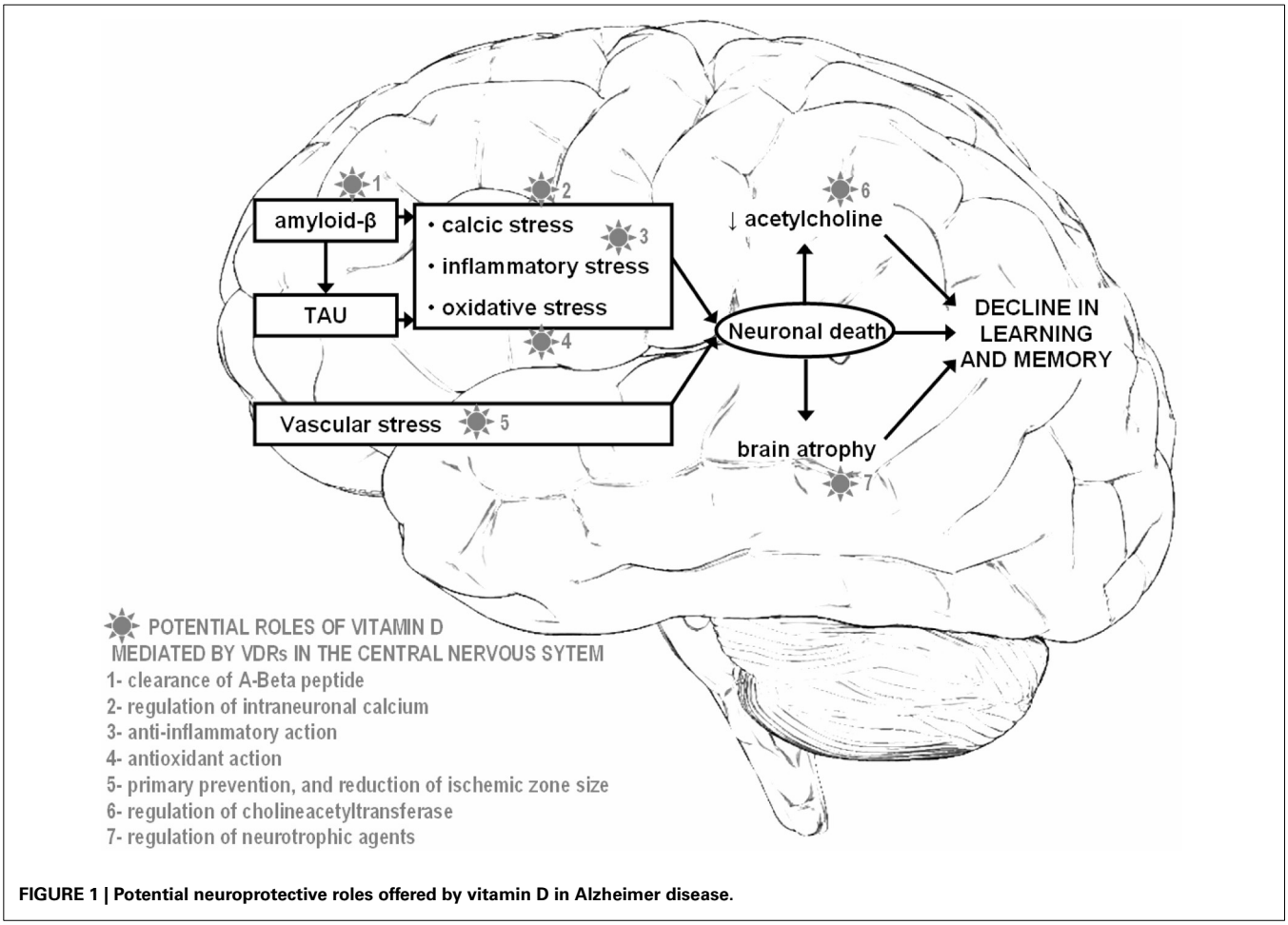

FIGURE 1 | Potential neuroprotective roles offered by vitamin D in Alzheimer disease.

to cultures enriched with memantine only or with vitamin D only (Annweiler et al., 2014).

CONCLUSIONS

In conclusion, AD is a public health concern due to its very high prevalence and because the few drugs currently available are only symptomatic. Current therapeutic research, which focuses mainly on curative disease-modifying treatments, addresses a very small fraction of AD patients, predominantly those with prodromal disease and no symptoms of dementia. It is thus necessary to consider the patients who already suffer from moderate-to-severe AD, who constitute the majority of AD patients. One of the best hopes for them may rely on the development of multi-target drugs. In particular, using vitamin D supplements as an adjunct to standard anti-dementia treatments appears to be a simple, inexpensive, and efficient therapeutic strategy and leads to encouraging prospects for the correction of neurological disorders in AD.

AUTHORS CONTRIBUTIONS

All authors meet all of the following criteria: (1) Contributing to the conception and design, or analyzing and interpreting data; (2) Drafting the article or revising it critically for important intellectual content; and (3) Approving the final version to be published.

REFERENCES

Aisen, P. S. (2010). Pre-dementia Alzheimer's trials: overview. *J. Nutr. Health Aging* 14, 294. doi: 10.1007/s12603-010-0065-2

Anand, R., Gill, K. D., and Mahdi, A. A. (2014). Therapeutics of Alzheimer's disease: past, present and future. *Neuropharmacology* 76, 27–50. doi: 10.1016/j.neuropharm.2013.07.004

Annweiler, C., Allali, G., Allain, P., Bridenbaugh, S., Schott, A. M., Kressig, R. W., et al. (2009). Vitamin D and cognitive performance in adults: a systematic review. *Eur. J. Neurol.* 16, 1083–1089. doi: 10.1111/j.1468-1331.2009.02755.x

Annweiler, C., and Beauchet, O. (2011). Vitamin D-mentia: randomized clinical trials should be the next step. *Neuroepidemiology* 37, 249–258. doi: 10.1159/000334177

Annweiler, C., and Beauchet, O. (2012). Possibility of a new anti-alzheimer's disease pharmaceutical composition combining memantine and vitamin D. *Drugs Aging* 29, 81–91. doi: 10.2165/11597550-000000000-00000

Annweiler, C., Brugg, B., Peyrin, J. M., Bartha, R., and Beauchet, O. (2014). Combination of memantine and vitamin D prevents axon degeneration induced by amyloid-beta and glutamate. *Neurobiol. Aging* 35, 331–335. doi: 10.1016/j.neurobiolaging.2013.07.029

Annweiler, C., Montero-Odasso, M., Llewellyn, D. J., Richard-Devantoy, S., Duque, G., and Beauchet, O. (2013a). Meta-analysis of memory and executive dysfunctions in relation to vitamin D. *J. Alzheimers Dis.* 37, 147–171. doi: 10.3233/JAD-130452

Annweiler, C., Llewellyn, D. J., and Beauchet, O. (2013b). Low serum vitamin D concentrations in Alzheimer's disease: a systematic review and meta-analysis. *J. Alzheimers Dis.* 33, 659–674. doi: 10.3233/JAD-2012-121432

Annweiler, C., Rolland, Y., Schott, A. M., Blain, H., Vellas, B., Herrmann, F. R., et al. (2012a). Higher

vitamin D dietary intake is associated with lower risk of Alzheimer's disease: a 7-year follow-up. *J. Gerontol. A Biol. Sci. Med. Sci.* 67, 1205–1211. doi: 10.1093/gerona/gls107

Annweiler, C., Fantino, B., Gautier, J., Beaudenon, M., Thiery, S., and Beauchet, O. (2012b). Cognitive effects of vitamin D supplementation in older outpatients visiting a memory clinic: a pre-post study. *J. Am. Geriatr. Soc.* 60, 793–795. doi: 10.1111/j.1532-5415.2011.03877.x

Annweiler, C., Herrmann, F. R., Fantino, B., Brugg, B., and Beauchet, O. (2012c). Effectiveness of the combination of memantine plus vitamin D on cognition in patients with Alzheimer disease: a pre-post pilot study. *Cogn. Behav. Neurol.* 25, 121–127. doi: 10.1097/WNN.0b013e31826df647

Annweiler, C., Schott, A. M., Berrut, G., Chauviré, V., Le Gall, D., Inzitari, M., et al. (2010). Vitamin D and ageing: neurological issues. *Neuropsychobiology* 62, 139–150. doi: 10.1159/000318570

Annweiler, C., Souberbielle, J. C., Schott, A. M., de Decker, L., Berrut, G., and Beauchet, O. (2011). Vitamin D in the elderly: 5 points to remember. *Geriatr. Psychol. Neuropsychiatr. Vieil.* 9, 259–267. doi: 10.1684/pnv.2011.0288

Birks, J. (2006). Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst. Rev.* CD005593. doi: 10.1002/14651858.CD005593

Cavalli, A., Bolognesi, M. L., Minarini, A., Rosini, M., Tumiatti, V., Recanatini, M., et al. (2008). Multi-target-directed ligands to combat neurodegenerative diseases. *J. Med. Chem.* 51, 347–372. doi: 10.1021/jm7009364

de Sant'Anna, M., and Morat, B. (2013). Non-drug-based management of Alzheimer's disease. *Soins Gerontol.* 102, 15–18.

Etgen, T., Sander, D., Bickel, H., Sander, K., and Förstl, H. (2012). Vitamin D deficiency, cognitive impairment and dementia: a systematic review and meta-analysis. *Dement. Geriatr. Cogn. Disord.* 33, 297–305. doi: 10.1159/000339702

Gezen-Ak, D., Dursun, E., Ertan, T., Hanagasi, H., Gürvit, H., Emre, M., et al. (2007). Association between vitamin D receptor gene polymorphism and Alzheimer's disease. *Tohoku J. Exp. Med.* 212, 275–282. doi: 10.1620/tjem.212.275

Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Johansson, P., Almqvist, E. G., Johansson, J. O., Mattsson, N., Andreasson, U., Hansson, O., et al. (2013). Cerebrospinal fluid (CSF) 25-hydroxyvitamin D concentration and CSF acetylcholinesterase activity are reduced in patients with Alzheimer's disease. *PLoS ONE* 8:e81989. doi: 10.1371/journal.pone.0081989

Kalueff, A. V., and Tuohimaa, P. (2007). Neurosteroid hormone vitamin D and its utility in clinical nutrition. *Curr. Opin. Clin. Nutr. Metab. Care* 10, 12–19. doi: 10.1097/MCO.0b013e328010ca18

Llewellyn, D. J., Lang, I. A., Langa, K. M., Muniz-Terrera, G., Phillips, C. L., Cherubini, A., et al. (2010). Vitamin D and risk of cognitive decline in elderly persons. *Arch. Intern. Med.* 170, 1135–1141. doi: 10.1001/archinternmed.2010.173

Matthews, F. E., Arthur, A., Barnes, L. E., Bond, J., Jagger, C., Robinson, L., et al. (2013). Medical Research Council Cognitive Function and Ageing Collaboration. A two-decade comparison of prevalence of dementia in individuals aged 65 years and older from three geographical areas of England: results of the Cognitive Function and Ageing Study I and II. *Lancet* 382, 1405–1412. doi: 10.1016/S0140-6736(13)61570-6

McShane, R., Areosa Sastre, A., and Minakaran, N. (2006). Memantine for dementia. *Cochrane Database Syst. Rev.* CD003154. doi: 10.1002/14651858.CD003154.pub5

Moon, M., Song, H., Hong, H. J., Nam, D. W., Cha, M. Y., Oh, M. S., et al. (2013). Vitamin D-binding protein interacts with Abeta and suppresses Abeta-mediated pathology. *Cell Death Differ.* 20, 630–638. doi: 10.1038/cdd.2012.161

Prybelski, R., Agrawal, S., Krueger, D., Engelke, J. A., Walbrun, F., and Binkley, N. (2008). Rapid correction of low vitamin D status in nursing home residents. *Osteoporos. Int.* 19, 1621–1628. doi: 10.1007/s00198-008-0619-x

Schrötter, A., Magraoui, F. E., Gröttrup, B., Wiltfang, J., Heinsen, H., Marcus, K., et al. (2013). Early diagnosis of neurodegenerative diseases—the long awaited Holy Grail and bottleneck of modern brain research—19th HUPO BPP workshop: May 22–24, 2013, Dortmund, Germany. *Proteomics* 13, 2938–2941. doi: 10.1002/pmic.201370164

Stein, M. S., Scherer, S. C., Ladd, K. S., and Harrison, L. C. (2011). A randomized controlled trial of high-dose vitamin D2 followed by intranasal insulin in Alzheimer's disease. *J. Alzheimers Dis.* 26, 477–484. doi: 10.3233/JAD-2011-110149

Sutherland, M. K., Somerville, M. J., Yoong, L. K., Bergeron, C., Haussler, M. R., and McLachlan, D. R. (1992). Reduction of vitamin D hormone receptor mRNA levels in Alzheimer as compared to Huntington hippocampus: correlation with calbindin-28k mRNA levels. *Brain Res. Mol. Brain Res.* 13, 239–250. doi: 10.1016/0169-328X(92)90032-7

Vellas, B., and Aisen, P. S. (2010). Early Alzheimer's trials: new developments. *J. Nutr. Health Aging* 14, 293. doi: 10.1007/s12603-010-0064-3

Wang, L., Hara, K., Van Baaren, J. M., et al. (2012). Vitamin D receptor and Alzheimer's disease: a genetic and functional study. *Neurobiol. Aging* 33, 1844.e1–1844.e9. doi: 10.1016/j.neurobiolaging.2011.12.038

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Cannabinoids for treatment of Alzheimer's disease: moving toward the clinic

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The limited effectiveness of current therapies against Alzheimer's disease (AD) highlights the need for intensifying research efforts devoted to developing new agents for preventing or retarding the disease process. During the last few years, targeting the endogenous cannabinoid system has emerged as a potential therapeutic approach to treat Alzheimer. The endocannabinoid system is composed by a number of cannabinoid receptors, including the well-characterized CB_1 and CB_2 receptors, with their endogenous ligands and the enzymes related to the synthesis and degradation of these endocannabinoid compounds. Several findings indicate that the activation of both CB_1 and CB_2 receptors by natural or synthetic agonists, at non-psychoactive doses, have beneficial effects in Alzheimer experimental models by reducing the harmful β-amyloid peptide action and tau phosphorylation, as well as by promoting the brain's intrinsic repair mechanisms. Moreover, endocannabinoid signaling has been demonstrated to modulate numerous concomitant pathological processes, including neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress. The present paper summarizes the main experimental studies demonstrating the polyvalent properties of cannabinoid compounds for the treatment of AD, which together encourage progress toward a clinical trial.

Keywords: cannabinoids, Alzheimer, CB_1 receptor, CB_2 receptor, β-amyloid, tau, oxidative stress, neuroinflammation

INTRODUCTION

Alzheimer is an age-dependent neurodegenerative process distinct from normal aging and characterized morphologically by the presence of senile plaques, mainly composed of different species of fibrillar β-amyloid (Aβ) produced by the cleavage of the Aβ precursor protein (APP) due to β- and γ-secretases, and by the presence of neurofibrillary tangles, mostly composed of various isoforms of hyper-phosphorylated and nitrated tau protein (Duyckaerts and Dickson, 2011; Ferrer, 2012). One tendency of opinion proposes that Aβ triggers plaque formation, tau hyper-phosphorylation, and disease progression (Hardy, 2009). This may happens in a percentage of familial Alzheimer's disease (AD) cases linked to mutations in genes encoding APP, and presenilin 1 and presenilin 2 which are enzymes involved in the cleavage of APP, or in Down syndrome (Bertram and Tanzi, 2011). However, tau hyper-phosphorylation precedes Aβ deposition in many cerebral regions in sporadic cases of AD (Braak and Braak, 1999).

Recent studies have shown that Aβ acts as a seed of new Aβ production and deposition under appropriate conditions (Frost and Diamond, 2010) and that abnormal tau promotes the production and deposition of hyper-phosphorylated tau under determinate experimental conditions (Clavaguera et al., 2009). Therefore, Aβ and hyper-phosphorylated tau promote the progression of the pathological process in an exponential way once these abnormal proteins are accumulated in the brain (Goedert et al., 2010; Ferrer, 2012).

In addition to these pathological hallmarks, multiple alterations converge in the pathogenesis of AD, including genetic and environmental factors. Vascular factors and concomitant pathologies worsen disease symptoms (Kovacs et al., 2008). Mitochondrial functional defects, increased production of reactive oxygen and nitrogen species (ROS and RNS), and damage to enzymes involved in energy metabolism are causative of nerve cell exhaustion (Pamplona et al., 2005; Ferrer, 2009; Sultana and Butterfield, 2010).

Altered lipid composition of membranes particularly lipid rafts (Martín et al., 2010), inflammatory responses (Akiyama et al., 2000), and altered production of trophic factors, neurotransmitter and neuromodulators, together with impaired function of degradation pathways such as those related to cytoplasmic proteolysis, autophagy, and ubiquitin–proteasome system play crucial roles as well (Keller et al., 2000; Ferrer, 2012; Selkoe, 2012).

Neurofibrillary tangles first appear in middle age in selected nuclei of the brain stem, later in the entorhinal cortex, and then extend to other parts of the brain (Braak and Braak, 1999; Simic et al., 2009). Senile plaques appear first in the orbitofrontal and temporal cortex and then extend to the whole cortex, diencephalic nuclei, and eventually to the cerebellum at terminal stages (Braak and Braak, 1999). Synaptic loss, reduced dendritic arbors, progressive isolation of remaining neurons and nerve cell loss occurs with disease progression, and affects multiple brain regions not only the cerebral cortex but also the amygdala, nuclei of the forebrain including

Meynert nucleus, striatum, thalamus, and selected nuclei of the brain stem thus involving multiple neurotransmitter systems (Duyckaerts and Dickson, 2011).

Importantly, the progression from early stages of the neurodegenerative process to symptomatic stages may take decades, whereas once the cognitive impairment and dementia appear the disease progression is much more rapid. Therefore, Alzheimer is a relatively well-tolerated degenerative process during a long period of time, but it may have devastating effects once thresholds are crossed (Ferrer, 2012). These facts highlight the need to search for treatments that act on selective targets during the silent period of the disease, aimed at curbing or retarding disease progression toward dementia (Ferrer, 2012; Selkoe, 2012).

During the last few years, targeting the endogenous cannabinoid system (ECS) has emerged as a potential therapeutic approach to treat Alzheimer in such first stages. Endocannabinoid signaling has been demonstrated to modulate the main pathological processes occurring during the silent period of the neurodegenerative process, including protein misfolding, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress. The present paper summarizes the experimental studies demonstrating the multi-faceted properties of cannabinoid compounds for the treatment of AD.

THE ENDOGENOUS CANNABINOID SYSTEM

The last two decades of research have brought a tremendous improvement in knowledge of the endocannabinoid system components and functions under physiological and pathological conditions. This neuromodulatory system consists of cannabinoid receptors, endogenous ligands, and several enzymes responsible for their synthesis and degradation (Piomelli, 2003). To date, two subtypes of cannabinoid $G_{i/o}$-coupled receptors, CB_1 and CB_2, have been fully characterized and cloned. However, cannabinoid compounds may also bind to other receptors, such as GPR55, peroxisome proliferator-activated receptors PPARα and PPARγ, and transient receptor potential vanilloid-1 (TRPV1) channels (Maccarrone et al., 2010; Pertwee et al., 2010). CB_1 receptors are the most abundant G protein-coupled receptors in the central neural system, expressed in both neurons and glial cells, where they regulate important brain functions including cognition and memory, emotion, motor control, feeding, and pain perception (Wilson and Nicoll, 2002; Howlett, 2005). CB_1 receptors are mostly located at the terminals of neurons of the central and peripheral nervous system where they act as modulators of excitatory and inhibitory neurotransmission. Moreover, CB_1 receptors are also found in peripheral tissues, playing an important role in energy balance and metabolism (Silvestri and Di Marzo, 2013). CB_2 receptors are localized in cells of the immune system and modulate the immune cell migration and the release of cytokines; within the nervous system CB_2 receptors are mainly located in microglia (Cabral and Griffin-Thomas, 2009). Relatively low CB_2 receptor expression has also recently been identified in some neurons (Van Sickle et al., 2005; Brusco et al., 2008; Onaivi et al., 2008). Further evidence of CB_2 receptor expression in neurons comes from the observation that axonal damage in one cerebellar hemisphere induced the expression of CB_2 receptors in contralateral precerebellar neurons; CB_2 receptor agonist facilitated neuronal survival, whereas the selective PI3K inhibitor blocked CB_2R effects on axotomized neurons (Viscomi et al., 2009). Most of the knowledge acquired about cannabinoid receptor pharmacology was made possible by the study of the mechanisms of action of numerous natural, but also synthetic, cannabinoid compounds. Among the natural cannabinoids, the most well-known are Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive compound of the *Cannabis sativa* plant, and cannabidiol (CBD), which is devoid of any psychoactivity, both differing in cannabinoid receptor affinity and activity (Pertwee, 2008).

The characterization of CB_1 and CB_2 receptors permitted the discovered of endocannabinoids or cannabinoids produced and released by nerve cells. Endocannabinoids are lipid compounds of the eicosanoid family derived from the degradation of membrane phospholipids (Piomelli, 2003). The most representative are arachidonoylethanolamine (AEA), also named anandamide, and 2-arachidonoylglycerol (2-AG), although several others have also been identified, such as 2-arachidonylglyceryl ether (2-AGE), virodhamine, and *N*-arachidonyldopamine (Piomelli, 2003). Endocannabinoids act as neurotransmitters since they are synthesized and released by neurons, are able to bind and activate membrane receptors, and are inactivated by reuptake and enzymatic degradation within the cell. However, endocannabinoids have two fundamental characteristics that differentiate them from other neurotransmitters: they act as retrograde messengers and they do not accumulate in the interior of synaptic vesicles (Wilson and Nicoll, 2002). For several years, endocannabinoid compounds have been supposed to be exclusively synthesized on demand to act on cells located near their site of synthesis, and then to be rapidly inactivated by the action of specific degradation enzymes. However, recent studies have shown intracellular stores of anandamide in places other than synaptic vesicles as in adiposomes where it is sequestered and concentrated to higher levels than in the extracellular space (Hillard and Jarrahian, 2000; Maccarrone et al., 2010). Moreover, an active intracellular binding of anandamide to TRPV1 and PPARs suggest an additional role of anandamide as a second messenger in intracellular signaling (Maccarrone et al., 2010). This new scenario contemplates the possibility that anandamide may act as an autocrine/paracrine ligand of CB receptors but also as an intracellular ligand to TRPV1 and PPARs; moreover, the presence of extracellular anandamide transporters would point anandamide as an endocrine messenger (Maccarrone et al., 2010).

Interestingly, neuronal damage can increase the production of endocannabinoids, which may provide a defense mechanism against toxicity (Stella et al., 1997; Marsicano et al., 2003). In the case of AEA, the synthesis is performed from the phosphatidylethanolamine existing in the cell membrane by the successive action of 2 enzymes, the *N*-acetyltransferase and phospholipase D (PLD). AEA action is determined by two processes that limit their availability, (i) transport from the synaptic cleft into the cell by passive diffusion or by a selective transporter, and (ii) the hydrolysis caused by two enzyme systems, mainly the fatty acid amide hydrolase (FAAH) but also monoacylglycerol lipase (MAGL; Di Marzo et al., 1994). 2-AG is the most abundant endogenous cannabinoid in the brain and its concentration

is about 200 times that of AEA (Stella et al., 1997). The formation of 2-AG is mediated by phospholipase C and diacylglycerol lipase (DAGL), and also produced on demand. In contrast to AEA, MAGL seems to be more involved in 2-AG degradation than FAAH (Dinh et al., 2002).

Interest in the role that ECS may play in neurodegenerative processes is based on findings revealing that the augmentation of cannabinoid tone contributes to brain homeostasis and neuron survival, suggesting that may offer protection against the deleterious consequences of pathogenic molecules.

THE ENDOGENOUS CANNABINOID SYSTEM IN AD BRAINS

The analysis of human *post-mortem* samples revealed some alterations in ECS composition and signaling in AD brains, although the bestowal of such modifications in the pathophysiology of the disease remains to be elucidated. The modifications described for CB_1 receptors in AD are ambiguous. Whereas some authors have reported a significant reduction in the CB_1 levels in cortical areas and in neurons distant from senile plaques (Ramírez et al., 2005; Solas et al., 2013), others have described no changes in the expression, distribution, or availability of CB_1 receptors in cortex and hippocampus in AD (Benito et al., 2003; Lee et al., 2010; Mulder et al., 2011; Ahmad et al., 2013) or have failed to dissociate CB_1 receptor expression changes from normal aging (Westlake et al., 1994). No correlation between CB_1 levels and any AD molecular marker or cognitive status has been found (Solas et al., 2013). In contrast, there is no controversy regarding the significant increase of CB_2 levels in AD brains, mainly corresponding to receptors expressed on microglia surrounding senile plaques (Ramírez et al., 2005; Solas et al., 2013). Interestingly, expression levels of CB_2 receptors correlates with $A\beta_{42}$ levels and plaque deposition, although not with cognitive status (Solas et al., 2013), suggesting that such pathogenic events induce CB_2 receptor expression. Additionally, both CB_1 and CB_2 cannabinoid receptors in the AD brain are nitrosylated, and this could contribute to the impaired coupling of these receptors to downstream effector signaling molecules (Ramírez et al., 2005).

A few studies addressed other components of ECS in AD human samples. The first study analyzing endocannabinoid levels reported no differences between AD patients and healthy controls in the plasmatic concentrations of AEA and 2-AG (Koppel et al., 2009). However, a recent lipidomic study in *post-mortem* brain samples revealed lower AEA levels in midfrontal and temporal cortices in AD compared to control subjects, which inversely correlated with the neurotoxic brain $A\beta_{42}$ peptide levels and cognitive deficiencies recorded in these patients, suggesting a contribution for $A\beta_{42}$-dependent AEA impairment to cognitive dysfunction (Jung et al., 2012). Moreover, some alterations have been found in the contents and/or activity of the enzymes related to endocannabinoid synthesis and degradation in AD brains. Thus, the endocannabinoid metabolizing enzyme FAAH is upregulated in AD both neuritic plaque-associated glia (Benito et al., 2003) and in peripheral blood mononuclear cells (D'Addario et al., 2012), and this could participate in the increase of AEA degradation in the vicinity of the senile plaque. Such FAAH overexpression may have at least two harmful consequences in disease progression, (i) neuronal AEA availability limitation and (ii) increase of pro-inflammatory molecules induced by AEA metabolites such as arachidonic acid (Calder, 2005). An elegant study revealed altered 2-AG signaling during late stages of AD due to the combination of impaired MAGL recruitment and increased DAGL levels, which subsidize synapse silencing in AD (Mulder et al., 2011). The same study failed to detect changes in PLD, FAAH, or TRPV1 protein levels in total hippocampal homogenates.

CLINICAL AND PRECLINICAL EVIDENCE OF THERAPEUTIC PROPERTIES OF CANNABINOIDS IN AD

Most of the evidence accumulated sustaining the potential therapeutic utility of cannabinoids in AD has been obtained by using cellular and animal models that mimic a variety of AD-related changes, and they will be discussed later on in this review. However, it is of note that the scarce clinical data available also support the beneficial effects of cannabinoid compounds for treating some behavioral symptoms related to AD. Only a few clinical trials and one case report are available on the topic so far. In all the cases an analog of Δ^9-THC (nabilone or dronabinol) was tested. Interestingly, one clinical trial including 15 AD patients resulted in a decreased severity of altered behavior and an increase in the body weight in AD patients, who were previously refusing food, after 6 weeks of dronabinol treatment. Side effects associated with cannabinoid administration were limited to euphoria, somnolence, and tiredness, but these did not warrant discontinuation of therapy (Volicer et al., 1997). Similarly, two pilot studies including eight patients with dementia concluded with a reduction in night-time agitation and behavioral disturbances, without adverse effects during the trial period with dronabinol (Walther et al., 2006, 2011). In line with these observations, the use of the cannabinoid receptor agonist nabilone correlated with prompt and dramatic improvements in the severe agitation and aggressiveness exhibited by an advanced AD patient who was refractory to antipsychotic and anxiolytic medications (Passmore, 2008). In spite of the low number of patients included in these trials and the fact that none of them evaluated cognitive or neurodegenerative markers, the positive behavioral results are promising and represent valuable, albeit limited, information, considering that no remarkable side effects were reported. However, the revision in 2009 of the Cochrane Dementia and Cognitive Improvement Group Specialized Register found no evidences of cannabinoid effectiveness in the improvement of behavior and other parameters of dementia, and suggested that more controlled trials are needed to assess the effectiveness of cannabinoids in the treatment of dementia (Krishnan et al., 2009).

EFFECT OF CANNABINOIDS ON Aβ

Several *in vitro* and *in vivo* studies demonstrate that certain cannabinoid compounds confer neuroprotection against Aβ, as previously reported elsewhere (Ruiz-Valdepeñas et al., 2010). Some endocannabinoids such as AEA, 2-AG, and noladin ether, directly supplied to the cell culture or augmenting their availability through administration of endocannabinoid reuptake inhibitors, increased the viability of neurons after exposure to different toxic Aβ species (Milton, 2002; Chen et al., 2011; Harvey et al.,

2012; Janefjord et al., 2013), and reduced Aβ-induced memory impairment in rats (van der Stelt et al., 2006). Similar positive results in the survival of neuronal cultures exposed to Aβ peptide were obtained with exogenous cannabinoids such as CBD (Iuvone et al., 2004; Janefjord et al., 2013), the selective CB_1 receptor agonist arachidonyl-2-chloroethylamide (ACEA; Aso et al., 2012), the CB_2 selective agonists JWH-015 and JWH-133, and the mixed CB_1/CB_2 receptor agonists Δ^9-THC, HU-210, and WIN55,212-2 (Ramírez et al., 2005; Janefjord et al., 2013). The neuroprotective properties of exogenous cannabinoids have consistently been demonstrated to prevent memory deficits in Aβ-injected rats and mice for both synthetic CB_1 (Haghani et al., 2012) and CB_2 selective agonists (Wu et al., 2013), as well as mixed CB_1/CB_2 receptor agonists (Ramírez et al., 2005; Martín-Moreno et al., 2011; Fakhfouri et al., 2012) and natural CBD (Martín-Moreno et al., 2011). Moreover, chronic treatment with ACEA (Aso et al., 2012), JWH-133 (Martín-Moreno et al., 2012; Aso et al., 2013), or WIN55,212-2 (Martín-Moreno et al., 2012) resulted in cognitive improvement in two different transgenic mouse models of brain amyloidosis. The efficacy of the cannabinoid compounds in curbing the cognitive impairment was inversely proportional to the disease progression stage at the beginning of the treatment in the transgenic animals (Aso et al., 2012, 2013).

The mechanisms of action that underlie the cannabinoid neuroprotection against Aβ, which ultimately may lead to the memory improvement, are multiple and are assumed to act in parallel or interacting within them. Although most of these proposed protective mechanisms are related to the capacity of cannabinoids to indirectly mitigate the harmful effects of Aβ, as we will discuss in later sections of this review (i.e., inflammation, oxidative stress, excitotoxicity, aberrant cellular signaling), some authors also described direct effects of cannabinoids on Aβ processing. Thus, stimulation of CB_2 receptors produced Aβ removal by human macrophages (Tolón et al., 2009; Wu et al., 2013) and favored Aβ transport through the choroid plexus (Martín-Moreno et al., 2012). This facilitation of Aβ clearance across the blood–brain barrier (BBB) was also demonstrated for the endocannabinoid 2-AG, a synthetic CB_1/CB_2 receptor agonist and MAGL inhibitors, but no FAAH, in *in vitro* an *in vivo* BBB models (Bachmeier et al., 2013). These findings could explain the reduction in Aβ levels and plaque burden observed in AD mouse models chronically treated with CB_2 or CB_1/CB_2 receptor agonists (Martín-Moreno et al., 2012) and MAGL inhibitors (Chen et al., 2012; Piro et al., 2012). In contrast, no significant contribution of CB_1 receptors in Aβ production, aggregation or clearance was reported after chronic treatment with ACEA (Aso et al., 2012) or HU-210 (Chen et al., 2010) in two different transgenic AD models. However, there is a study reporting a regulatory influence of CB_1 receptor on APP processing since APP23 transgenic mice deficient for CB_1 receptor exhibited reduced APP protein levels and Aβ plaque deposition, likely due to changes in intracellular APP transport, although the animals presented enhanced cognitive deficits (Stumm et al., 2013). Moreover, a recent publication revealed that Δ^9-THC significantly increased the expression of neprilysin, an important endopeptidase for Aβ degradation, but not β-site APP cleaving enzyme 1 (BACE1), leading to a remarkable reduction of Aβ plaques in 5xFAD APP transgenic mice (Chen et al., 2013). This study failed, however, to clarify the specific role of CB_1 or CB_2 receptors in such Δ^9-THC effect on Aβ clearance.

CANNABINOIDS ON TAU HYPER-PHOSPHORYLATION

A role for cannabinoids in another AD pathological hallmark, tau hyper-phosphorylation, has also been described. First studies performed in cell cultures demonstrated that CBD, ACEA, and WIN55,212-2 inhibited tau protein hyper-phosphorylation in Aβ-stimulated PC12 neuronal cells (Esposito et al., 2006a,b). In the case of CBD, the effect was mediated through the reduction of the phosphorylated active form of glycogen synthase kinase 3β (GSK-3β), one of the known tau kinases (Ferrer et al., 2005), which in turn resulted in Wnt/β-catenin pathway rescue and consequent reduction of neuronal apoptosis (Esposito et al., 2006a). In contrast, the ACEA and WIN55,212-2 effect on tau hyper-phosphorylation was selectively mediated by CB_1 receptor through the down-regulation of inducible nitric oxide synthase (iNOS) protein expression and nitric oxide (NO) production in Aβ-stimulated astroglioma cells co-cultured with the PC12 neuronal cells (Esposito et al., 2006b). In line with the described role for CB_1 receptor on tau hyper-phosphorylation, chronic treatment with the CB_1 selective agonist ACEA reduced the levels of tau phosphorylated at Thr181 site in the area surrounding Aβ plaques in treated APP/PS1 mice, probably through the ACEA-induced reduction in GSK-3β harmful activity (Aso et al., 2012). Moreover, a specific role for CB_2 receptor in the modulation of tau phosphorylation was also suggested. Chronic JWH-133 administration reduced tau hyper-phosphorylation in the vicinity of Aβ plaques in APP/PS1 mice, which may be explained by decreased activity of GSK-3β, p38, and stress-activated protein kinase/c-Jun NH(2)-terminal kinase (SAPK/JNK) kinases in the treated animals (Aso et al., 2013).

Confirming these observations, a recent study reported a marked reduction in neurofibrillary tangles in parkin-null, human tau overexpressing ($PK^{-/-}$/TauVLW) mice, a model of complex frontotemporal dementia, parkinsonism, and lower motor neuron disease, after prolonged exposure to Sativex®, an already approved medicine based on mixed Δ^9-THC and CBD natural extracts (Casarejos et al., 2013). The authors suggested the cannabinoid potentiation of autophagy or improvement of redox state as likely mechanisms accounting for the reduction in tau deposition.

ANTI-INFLAMMATORY PROPERTIES OF CANNABINOIDS

Neuroinflammation, initially manifested as microglial activation, is a prominent feature in AD which contributes to progressive cell damage and neuron loss (Akiyama et al., 2000; Hensley, 2010; Sardi et al., 2011). As CB_2 receptors are essentially expressed in the immune system including microglial cells, where they are known to inhibit microglia-mediated neurotoxicity, the main interest in the role of cannabinoids as anti-inflammatory agents in several diseases concurring with inflammation has focused on compounds acting on CB_2 receptors (Cabral and Griffin-Thomas, 2009). In the case of AD, several studies reported that activation of CB_2 receptors reduced the neuroinflammatory response to Aβ insults in different models of the disease. After the inoculation of Aβ into the rat or mouse brain, selective or

mixed CB_2 receptor agonists reduced microglial response and pro-inflammatory molecule production in a plethora of studies (Ramírez et al., 2005; van der Stelt et al., 2006; Esposito et al., 2007; Fakhfouri et al., 2012; Wu et al., 2013). Similarly, selective CB_2 receptor agonists decreased the number of activated microglial cells surrounding Aβ deposition and the levels of pro-inflammatory cytokines in at least two APP transgenic models (Martín-Moreno et al., 2012; Aso et al., 2013). Moreover, the Δ^9-THC and CBD natural mixture present in Sativex® also blunted the microglial reactivity in a genetic tauopathy model (Casarejos et al., 2013), although no evidence of direct implication of CB_2 receptors or other receptors in such effects was provided. In fact, other mechanisms related to ECS components distinct from CB_2 receptors could explain the anti-inflammatory effects of the Sativex® preparation. As noted above, Δ^9-THC is a partial agonist of CB_1 receptors, which could also play a role in the AD inflammatory process according to a recent study demonstrating that chronic treatment with the selective agonist ACEA reduced the astrocytic expression of the pro-inflammatory cytokine interferon-γ in APP/PS1 transgenic mice (Aso et al., 2012). Additionally, CBD, which has no affinity for CB_1 or CB_2 receptors, also presents anti-inflammatory properties in AD models (Esposito et al., 2006a; Martín-Moreno et al., 2011). The precise site at which CBD could exert its neuroinflammatory and neuroprotective effects is still not fully elucidated, but some findings point to the selective involvement of PPARγ in such CBD properties (Esposito et al., 2011).

The enzymes related to AEA and 2-AG degradation also contribute to modulating the inflammatory process in AD models. FAAH is expressed in both neurons and astrocytes, where it may play a role in the response to inflammation. In fact, an astrocyte-specific increase in FAAH expression is markedly maintained in neuroinflammatory conditions including amyloidosis, which was assumed to contribute to the harmful processes induced by toxic insults because of the reduction in endocannabinoid tone (Benito et al., 2003). However, cortical mouse astrocytes genetically modified to lack FAAH exhibited a pro-inflammatory phenotype when exposed to Aβ, characterized by an increase in cytokine concentration and cell death probably due to the modification of signaling cascades involved in cell survival and inflammation, such as extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (p38MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as well as to the increase in inflammatory mediators such as iNOS and cyclooxygenase (COX-2; Benito et al., 2012). The authors demonstrated that these processes involved PPAR-α, PPAR-γ, and TRPV1, but not CB_1 or CB_2 receptors. Yet, the pharmacological blockade of FAAH in cell cultures did not lead astrocytes to a pro-inflammatory phenotype, indicating that the observed effects in astrocytes lacking FAAH could be due to compensatory changes that result from the potentially prolonged enhancement of *N*-acylethanolamines. These data suggest that an excessively prolonged enhancement of the endocannabinoid tone may have harmful consequences. In contrast, the genetic inactivation of MAGL, an enzyme known to hydrolyze endocannabinoids and generate the primary arachidonic acid pool for neuroinflammatory prostaglandins (Nomura et al., 2011), attenuated neuroinflammation and lowered Aβ levels and plaques in APP/PS1 mice (Piro et al., 2012). These observations were confirmed by the pharmacological blockade of MAGL, which recapitulated the cytokine-lowering effects through reduced prostaglandin production, rather than enhanced endocannabinoid signaling.

CANNABINOID ACTIONS ON MITOCHONDRIA ACTIVITY: OXIDATIVE STRESS AND ENERGY METABOLISM

Mitochondria are vital cellular components essential for ATP production and calcium homeostasis. The relevance of these organelles in neurons is even greater than in other cell types because neurons are highly demanding energy cells mainly dependent on aerobic oxidative phosphorylation, due to their limited capacity for glycolysis. Long axons require energy transport over long distances, and synaptic transmission depends on calcium signals. Mitochondria are abundant in presynaptic nerve terminals where they provide energy for sustained neurotransmitter release. Therefore, defects in mitochondrial activity can have severe consequences for the cell, including energetic failure associated with decreased ATP production and apoptosis resulting from the release of death factors and impaired calcium-buffering capacity. Moreover, alterations in the protein complexes of the respiratory chain located in the inner mitochondrial membrane lead to electron transport leakage that enables the production of ROS, which may overwhelm the capacity of the anti-oxidant systems existing in cells to counteract free radical damage, with the subsequent oxidative damage produced to proteins, DNA, RNA, and lipids. Numerous studies have linked mitochondrial dysfunction to neurodegenerative diseases, including AD (Ferrer, 2009; Ankarcrona et al., 2010; Burchell et al., 2010). Altered mitochondrial function appears early in time in AD, even preceding the characteristic Alzheimer pathology in mouse models, and ultimately leads to exhausted neurons as a result of the convergence of reduced energy production, increased energy demand, and excessive oxidative stress (Ferrer, 2009).

The anti-oxidant properties of cannabis derivatives, notably CBD, were demonstrated early in cell cultures exposed to toxic glutamate levels (Hampson et al., 1998, 2000). In line with these findings, CBD prevented ROS production and lipid peroxidation in PC12 neuronal cells exposed to Aβ, as well as reduced apoptosis from reduced caspase 3 levels, and in counteracting the Aβ-induced increase in intracellular calcium concentration (Iuvone et al., 2004). CBD also reduced, in similar conditions, the levels of nitrite (NO), a potent oxidant reactive molecule, as well as the expression of iNOS, one of the enzymes responsible for the synthesis of NO (Esposito et al., 2006b, 2011). Moreover, other cannabinoid compounds exhibited anti-oxidant properties in animal models of AD. Thus, the selective CB_2 receptor agonist JWH-133 reduced hydroxynonenal adducts, derived from lipid peroxidation, and enhanced the levels of the superoxide dismutases SOD1 and SOD2 in the vicinity of plaques in APP/PS1 mice, indicating the role of CB_2 receptors in reducing oxidative stress damage and to promote responses against such damage (Aso et al., 2013). A reduction of free radicals and mitochondrial activity was also suggested in a mouse model of tauopathy exposed to

chronic treatment with the Sativex® mixture of Δ^9-THC and CBD (Casarejos et al., 2013).

An additional topic deserving attention is the putative role of cannabinoid receptors in the regulation of neuronal energy metabolism. The little information available to date supports the direct control of CB_1 over neuronal respiration and energy production. One study, using anti-CB_1 receptor antibodies, revealed CB_1 receptor protein localization in approximately 30% of neuronal mitochondria, which when activated by exogenous or endogenous cannabinoids reduces the respiratory chain complex I activity and oxygen consumption, likely through cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) signaling (Bénard et al., 2012). These findings are in agreement with previous observations showing that AEA, Δ^9-THC, and HU-210, all of them partial CB_1 agonists, significantly decreased oxygen consumption and mitochondrial membrane potential (Athanasiou et al., 2007). However, these data must be interpreted with caution as commercial anti-CB_1 receptor antibodies also recognize the mitochondrial protein stomatin-like protein 2 and that the formerly described effect of WIN 55,212-2 on mitochondrial complex III is in fact not detectable in isolated mitochondrial preparations (Morozov et al., 2013).

CANNABINOIDS MODULATE NEUROTRANSMISSION

Nowadays the approved drugs for treating AD are based on acetylcholine esterase (AChE) inhibitors, which increase the ACh availability partially palliating this neurotransmitter deficiency in AD patients, or they are non-competitive antagonists of the *N*-methyl D-aspartate (NMDA) receptor, which reduce calcium influx and limit excitotoxicity. Interestingly, certain cannabinoid compounds act on the same targets than current medications, resulting in similar or enhanced beneficial effects. For instance, Δ^9-THC competitively inhibits AChE, thus increasing ACh levels, as well as preventing AChE-induced Aβ aggregation by binding in the peripheral anionic site of AChE, the critical region involved in amyloidogenesis (Eubanks et al., 2006). The synthetic cannabinoid HU-211 acts as a stereoselective inhibitor of NMDA receptors, and thus protects cells from NMDA induced neurotoxicity (Feigenbaum et al., 1989; Eshhar et al., 1993; Nadler et al., 1993). In the case of HU-211, its neuroprotective activity is due to the direct binding to NMDA receptors, not to cannabinoid receptors, but the broadly accepted cannabinoid-mediated neuroprotection against excitotoxicity can be achieved through a number of other different mechanisms, including inhibition of presynaptic glutamate release (Marsicano et al., 2003), blockage of voltage-dependent calcium channels (Mackie and Hille, 1992; Twitchell et al., 1997) and inhibition of calcium release from ryanodine sensitive stores (Zhuang et al., 2005), which mostly imply the direct or indirect participation of CB_1 receptors.

OTHER EFFECTS OF CANNABINOIDS IN AD

Several other mechanisms seem to contribute to the therapeutic properties of cannabinoid compounds in AD, although they have not been fully characterized. Among them, we can note the capacity of endocannabinoids to prevent Aβ-mediated lysosomal destabilization in cultured neurons, reducing in this way the apoptotic signaling, which in turn sustains cell survival (Noonan et al., 2010). Compromised neurogenesis is an early event in AD that limits neuronal replacement once progressive neuronal loss takes place in the brain, contributing to cognitive deterioration (Lazarov and Marr, 2010). Interestingly, AEA and CBD have been described as promoting neurogenesis in response to Aβ insult (Esposito et al., 2011; Tanveer et al., 2012), suggesting an additional beneficial effect of cannabinoids in AD.

Another aspect as yet unexplored is the interaction of cannabinoids with neurotrophic factors in AD. Cannabinoids are capable of increasing brain-derived neurotrophic factor (BDNF; Khaspekov et al., 2004), a neurotrophin reduced in the AD brain (Lee et al., 2005; Peng et al., 2009), which is known to confer protection against excitotoxicity and to promote neurogenesis (Scharfman et al., 2005) and neuronal plasticity; all of these processes play a role in AD. However, there is still no evidence about the implication that such cannabinoid-induced BDNF promotion could have on the cognitive or pathological aspects of AD. Similarly, little is known about the participation of cannabinoid signaling in the impaired function of degradation pathways such as autophagy and ubiquitin–proteasome, which are known to play a relevant role in AD progression. The impairment in these catabolic processes results in accumulation of aggregate-prone proteins, altered mitochondria and other cellular organelles that might exacerbate neurodegenerative process (Nixon et al., 2005; Harris and Rubinsztein, 2011). To date, only one study has reported beneficial effects of cannabinoid-induced autophagy in a model of tauopathy (Casarejos et al., 2013), but this has opened the possibility of exploring in greater detail the involvement of ECS in promoting degradation of toxic components in neurodegenerative diseases. Finally, the effect of cannabinoids on the regulation of cerebral blood flow may contribute to their potential benefits on AD. A number of studies have demonstrated that certain cannabinoids produce vasodilatation of brain blood vessels and increase cerebral blood flow (Ellis et al., 1995; Wagner et al., 2001; Pacher et al., 2005; Iring et al., 2013). Considering that cerebral blood flow in AD contributes to the reduction of oxygen and nutrients in brain (Iadecola, 2004), it can be suggested that treatments improving cerebral perfusion such as cannabinoids are advantageous in AD. Taken together, available information suggest that cannabinoids may have multiple effects on AD by acting not only as anti-oxidant and anti-inflammatory agents, but also modulating a plethora of factors which contribute to the pathogenesis of AD as altered Aβ metabolism, autophagy, trophic factor deficiencies, and impaired blood flow.

CONCLUSIONS AND THERAPEUTIC IMPLICATIONS

Considering the numerous complex pathological mechanisms involved in the progression of AD, treatments targeting a single causal or modifying factor offer limited benefit. Cannabinoids, however, exhibit pleiotropic activity, targeting in parallel several processes that play key roles in AD, including Aβ and tau aberrant processing, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress. Cannabinoids improve behavioral disturbances, as well. These effects are summarized in

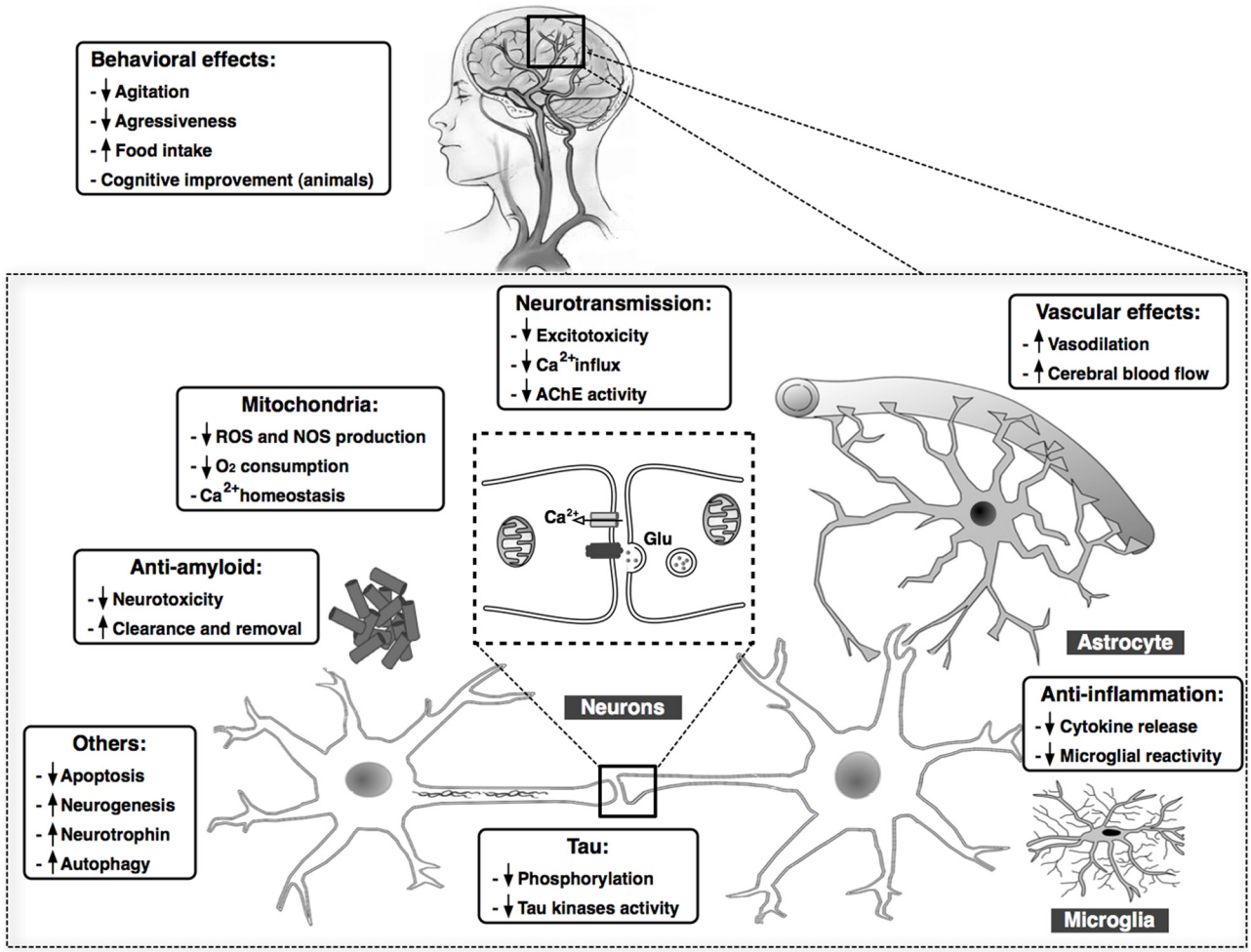

FIGURE 1 | Summary of the main findings demonstrating beneficial effects of cannabinoid compounds in AD models. Cannabinoids may target in parallel several processes that play key roles in AD, including Aβ and tau aberrant processing, chronic inflammatory responses, excitotoxicity, mitochondrial dysfunction, and oxidative stress, among others. Clinical data also reveal an improvement in behavioral in patients with AD after treatment with cannabinoids.

Figure 1. Then, because of these widespread properties of cannabinoid compounds, targeting the ECS could represent a unique and reliable opportunity to advance toward an effective therapy against the AD. Moreover, cannabinoids might represent a safe low-cost therapy, with their natural origin and low side effects profile. From our point of view, the success of cannabinoid-based therapy in AD could be increased taking into account two important aspects: (i) the use of a combination of compounds that cover the whole spectrum of therapeutic properties described for cannabinoids, i.e., combination of CB_1 and CB_2 receptors agonists plus CBD, which presents interesting neuroprotective properties spite of its mechanism of action remaining poorly understood, and (ii) the early initiation of the treatment in the neurodegenerative process, which ensures the integrity of the ECS target components and increases the possibility of curbing the exponential degenerative progression toward dementia.

The main concerns regarding the use of cannabis derivatives in medicine are related with the psychoactivity of some cannabinoids, especially Δ^9-THC, which may disrupt short-term memory, working memory, and attention skills mainly acting through CB_1 receptors, as well as with the potential Δ^9-THC dependence occurring after long-term use. However, the therapeutic effects of cannabinoids must be clearly dissociated from the risks of abuse and addiction linked to the recreational use of cannabis derivatives. First, the CB_1 agonists with potential psychoactivity used in experimental models to demonstrate the therapeutic properties were administered at doses substantially lower than those producing psychoactive effects and cannabis dependence (Maldonado et al., 2011). Second, the preferred therapeutic cannabinoid combination includes CBD, which is known to mitigate the negative consequences on cognition of Δ^9-THC administration (Fadda et al., 2004), and therefore insure the avoidance of such undesirable effects. Finally, the brain context in healthy subjects consuming cannabis enriched in Δ^9-THC for recreational purposes is completely different from that of AD patients subjected to very determined combinations of cannabinoid species, in

terms of ECS organization and neuronal signaling. In conclusion, in light of the polyvalent properties for the treatment of AD and the limited side effects exhibited by these compounds, progress toward a clinical trial to test the capacity of cannabinoids to curb this neurodegenerative disease seems to be fully justified.

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REFERENCES

Ahmad, R., Goffin, K., Van den Stock, J., De Winter, F. L., Cleeren, E., Bormans, G., et al. (2013). In vivo type 1 cannabinoid receptor availability in Alzheimer's disease. *Eur. Neuropsychopharmacol.* 24, 242–250. doi: 10.1016/j.euroneuro.2013.10.002

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., et al. (2000). Inflammation and Alzheimer disease. *Neurobiol. Aging* 21, 383–421. doi: 10.1016/S0197-4580(00)00124-X

Ankarcrona, M., Mangialasche, F., and Winblad, B. (2010). Rethinking Alzheimer's disease therapy: are mitochondria the key? *J. Alzheimers Dis.* 20(Suppl. 2), S579–S590. doi: 10.3233/JAD-2010-100327

Aso, E., Juvés, S., Maldonado, R., and Ferrer, I. (2013). CB2 cannabinoid receptor agonist ameliorates Alzheimer-like phenotype in AβPP/PS1 mice. *J. Alzheimers Dis.* 35, 847–858. doi: 10.3233/JAD-130137

Aso, E., Palomer, E., Juvés, S., Maldonado, R., Muñoz, F. J., and Ferrer, I. (2012). CB1 agonist ACEA protects neurons and reduces the cognitive impairment of AβPP/PS1 mice. *J. Alzheimers Dis.* 30, 439–459. doi: 10.3233/JAD-2012-111862

Athanasiou, A., Clarke, A. B., Turner, A. E., Kumaran, N. M., Vakilpour, S., Smith, P. A., et al. (2007). Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. *Biochem. Biophys. Res. Commun.* 364, 131–137. doi: 10.1016/j.bbrc.2007.09.107

Bachmeier, C., Beaulieu-Abdelahad, D., Mullan, M., and Paris, D. (2013). Role of the cannabinoid system in the transit of beta-amyloid across the blood–brain barrier. *Mol. Cell. Neurosci.* 56, 255–262. doi: 10.1016/j.mcn.2013.06.004

Bénard, G., Massa, F., Puente, N., Lourenço, J., Bellocchio, L., Soria-Gómez, E., et al. (2012). Mitochondrial CB1 receptors regulate neuronal energy metabolism. *Nat. Neurosci.* 15, 558–564. doi: 10.1038/nn.3053

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., et al. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *J. Neurosci.* 23, 11136–11141.

Benito, C., Tolón, R. M., Castillo, A. I., Ruiz-Valdepeñas, L., Martínez-Orgado, J. A., Fernández-Sánchez, F. J., et al. (2012). β-Amyloid exacerbates inflammation in astrocytes lacking fatty acid amide hydrolase through a mechanism involving PPAR-α, PPAR-γ and TRPV1, but not CB1 or CB2 receptors. *Br. J. Pharmacol.* 166, 1474–1489. doi: 10.1111/j.1476-5381.2012.01889.x

Bertram, L., and Tanzi, R. E. (2011). "Genetics of Alzheimer's disease," in *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders*, 2nd Edn, eds D. Dickson and R. Weller (West Sussex: Wiley-Blackwell), 51–91. doi: 10.1002/9781444341256.ch9

Braak, H., and Braak, E. (1999). "Temporal sequence of Alzheimer's disease-related pathology," in *Cerebral Cortex*, Vol. 14, *Neurodegenerative and Age-related Changes in Structure and Function of Cerebral Cortex*, eds A. Peters and J. H. Morrison (New York: Kluwer Academic/Plenum Publishers), 475–512. doi: 10.1007/978-1-4615-4885-0_14

Brusco, A., Tagliaferro, P. A., Saez, T., and Onaivi, E. S. (2008). Ultrastructural localization of neuronal brain CB2 cannabinoid receptors. *Ann. N. Y. Acad. Sci.* 1139, 450–457. doi: 10.1196/annals.1432.037

Burchell, V. S., Gandhi, S., Deas, E., Wood, N. W., Abramov, A. Y., and Plun-Favreau, H. (2010). Targeting mitochondrial dysfunction in neurodegenerative disease: part I. *Expert Opin. Ther. Targets* 14, 369–385. doi: 10.1517/14728221003652489

Cabral, G. A., and Griffin-Thomas, L. (2009). Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. *Expert Rev. Mol. Med.* 11:e3. doi: 10.1017/S1462399409000957

Calder, P. C. (2005). Polyunsaturated fatty acids and inflammation. *Biochem. Soc. Trans.* 33, 423–427. doi: 10.1042/BST0330423

Casarejos, M. J., Perucho, J., Gómez, A., Muñoz, M. P., Fernández-Estévez, M., Sagredo, O., et al. (2013). Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. *J. Alzheimers Dis.* 35, 525–539. doi: 10.3233/JAD-130050

Chen, B., Bromley-Brits, K., He, G., Cai, F., Zhang, X., and Song, W. (2010). Effect of synthetic cannabinoid HU210 on memory deficits and neuropathology in Alzheimer's disease mouse model. *Curr. Alzheimer Res.* 7, 255–261. doi: 10.2174/156720510791050948

Chen, R., Zhang. J., Fan, N., Teng, Z. Q., Wu, Y., Yang, H., et al. (2013). Δ(9)-THC-caused synaptic and memory impairments are mediated through COX-2 signaling. *Cell* 155, 1154–1165. doi: 10.1016/j.cell.2013.10.042

Chen, R., Zhang, J., Wu, Y., Wang, D., Feng, G., Tang, Y. P., et al. (2012). Monoacylglycerol lipase is a therapeutic target for Alzheimer's disease. *Cell Rep.* 2, 1329–1339. doi: 10.1016/j.celrep.2012.09.030

Chen, X., Zhang, J., and Chen, C. (2011). Endocannabinoid 2-arachidonoylglycerol protects neurons against β-amyloid insults. *Neuroscience* 178, 159–168. doi: 10.1016/j.neuroscience.2011.01.024

Clavaguera, F., Bolmont, T., Crowther, R. A., Abramowski, D., Frank, S., Probst, A., et al. (2009). Transmission and spreading of tauopathy in transgenic mouse brain. *Nat. Cell Biol.* 11, 909–913. doi: 10.1038/ncb1901

D'Addario, C., Di Francesco, A., Arosio, B., Gussago, C., Dell'Osso, B., Bari, M., et al. (2012). Epigenetic regulation of fatty acid amide hydrolase in Alzheimer disease. *PLoS ONE* 7:e39186. doi: 10.1371/journal.pone.0039186

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., et al. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* 372, 686–691. doi: 10.1038/372686a0

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., et al. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10819–10824. doi: 10.1073/pnas.152334899

Duyckaerts, C., and Dickson, D. (2011). "Neuropathology of Alzheimer's disease and its variants," in *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders*, 2nd Edn, eds D. Dickson and R. Weller (West Sussex, Wiley-Blackwell), 62–91.

Ellis, E. F., Moore, S. F., and Willoughby, K. A. (1995). Anandamide and delta 9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am. J. Physiol.* 269, H1859–H1864.

Eshhar, N., Striem, S., and Biegon, A. (1993). HU-211, a non-psychotropic cannabinoid, rescues cortical neurones from excitatory amino acid toxicity in culture. *Neuroreport* 5, 237–240. doi: 10.1097/00001756-199312000-00013

Esposito, G., De Filippis, D., Carnuccio, R., Izzo, A. A., and Iuvone, T. (2006a). The marijuana component cannabidiol inhibits beta-amyloid-induced tau protein hyperphosphorylation through Wnt/beta-catenin pathway rescue in PC12 cells. *J. Mol. Med.* 84, 253–258. doi: 10.1007/s00109-005-0025-1

Esposito, G., De Filippis, D., Steardo, L., Scuderi, C., Savani, C., Cuomo, V., et al. (2006b). CB1 receptor selective activation inhibits beta-amyloid-induced iNOS protein expression in C6 cells and subsequently blunts tau protein hyperphosphorylation in co-cultured neurons. *Neurosci. Lett.* 404, 342–346. doi: 10.1016/j.neulet.2006.06.012

Esposito, G., Iuvone, T., Savani, C., Scuderi, C., De Filippis, D., Papa, M., et al. (2007). Opposing control of cannabinoid receptor stimulation on amyloid-beta-induced reactive gliosis: in vitro and in vivo evidence. *J. Pharmacol. Exp. Ther.* 322, 1144–1152. doi: 10.1124/jpet.107.121566

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., Latina, V., De Filippis, D., et al. (2011). Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS ONE* 6:e28668. doi: 10.1371/journal.pone.0028668

Eubanks, L. M., Rogers, C. J., Beuscher, A. E. IV, Koob, G. F., Olson, A. J., Dickerson, T. J., et al. (2006). A molecular link between the active component of marijuana and Alzheimer's disease pathology. *Mol. Pharm.* 3, 773–777. doi: 10.1021/mp060066m

Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G., and Riedel, G. (2004). Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats. *Neuropharmacology* 47, 1170–1179. doi: 10.1016/j.neuropharm.2004.08.009

Fakhfouri, G., Ahmadiani, A., Rahimian, R., Grolla, A. A., Moradi, F., and Haeri, A. (2012). WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway. *Neuropharmacology* 63, 653–666. doi: 10.1016/j.neuropharm.2012.05.013

Feigenbaum, J. J., Bergmann, F., Richmond, S. A., Mechoulam, R., Nadler, V., Kloog, Y., et al. (1989). Nonpsychotropic cannabinoid acts as a functional *N*-methyl-D-aspartate receptor blocker. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9584–9587. doi: 10.1073/pnas.86.23.9584

Ferrer, I. (2009). Altered mitochondria, energy metabolism, voltage-dependent anion channel, and lipid rafts converge to exhaust neurons in Alzheimer's disease. *J. Bioenerg. Biomembr.* 41, 425–431. doi: 10.1007/s10863-009-9243-5

Ferrer, I. (2012). Defining Alzheimer as a common age-related neurodegenerative process not inevitably leading to dementia. *Prog. Neurobiol.* 97, 38–51. doi: 10.1016/j.pneurobio.2012.03.005

Ferrer, I., Gómez-Isla, T., Puig, B., Freixes, M., Ribé, E., Dalfó, E., et al. (2005). Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr. Alzheimer Res.* 2, 3–18. doi: 10.2174/1567205052772713

Frost, B., and Diamond, M. I. (2010). Prion-like mechanisms in neurodegenerative diseases. *Nat. Rev. Neurosci.* 11, 155–159. doi: 10.1038/nrn2786

Goedert, M., Clavaguera, F., and Tolnay, M. (2010). The propagation of prion-like protein inclusions in neurodegenerative diseases. *Trends Neurosci.* 33, 317–325. doi: 10.1016/j.tins.2010.04.003

Haghani, M., Shabani, M., Javan, M., Motamedi, F., and Janahmadi, M. (2012). CB1 cannabinoid receptor activation rescues amyloid β-induced alterations in behaviour and intrinsic electrophysiological properties of rat hippocampal CA1 pyramidal neurones. *Cell Physiol. Biochem.* 29, 391–406. doi: 10.1159/000338494

Hampson, A. J., Grimaldi, M., Axelrod, J., and Wink, D. (1998). Cannabidiol and (−) D9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8268–8273. doi: 10.1073/pnas.95.14.8268

Hampson, A. J., Grimaldi, M., Lolic, M., Wink, D., Rosenthal, R., and Axelrod, J. (2000). Neuroprotective antioxidants from marijuana. *Ann. N.Y. Acad. Sci.* 899, 274–282. doi: 10.1111/j.1749-6632.2000.tb06193.x

Hardy, J. (2009). The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J. Neurochem.* 110, 1129–1134. doi: 10.1111/j.1471-4159.2009.06181.x

Harris, H., and Rubinsztein, D. C. (2011). Control of autophagy as a therapy for neurodegenerative disease. *Nat. Rev. Neurol.* 8, 108–117. doi: 10.1038/nrneurol.2011.200

Harvey, B. S., Ohlsson, K. S., Mååg, J. L., Musgrave, I. F., and Smid, S. D. (2012). Contrasting protective effects of cannabinoids against oxidative stress and amyloid-β evoked neurotoxicity in vitro. *Neurotoxicology* 33, 138–146. doi: 10.1016/j.neuro.2011.12.015

Hensley, K. (2010). Neuroinflammation in Alzheimer's disease: mechanisms, pathologic consequences, and potential for therapeutic manipulation. *J. Alzheimers Dis.* 21, 1–14. doi: 10.3233/JAD-2010-1414

Hillard, C. J., and Jarrahian, A. (2000). The movement of N-arachidonoylethanolamine (anandamide) across cellular membranes. *Chem. Phys. Lipids* 108, 123–134. doi: 10.1016/S0009-3084(00)00191-2

Howlett, A. C. (2005). Cannabinoid receptor signaling. *Handb. Exp. Pharmacol.* 168, 53–79. doi: 10.1007/3-540-26573-2_2

Iadecola, C. (2004). Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat. Rev. Neurosci.* 5, 347–360. doi: 10.1038/nrn1387

Iring, A., Ruisánchez, É., Leszl-Ishiguro, M., Horváth, B., Benkō, R., Lacza, Z., et al. (2013). Role of endocannabinoids and cannabinoid-1 receptors in cerebrocortical blood flow regulation. *PLoS ONE* 8:e53390. doi: 10.1371/journal.pone.0053390

Iuvone, T., Esposito, G., Esposito, R., Santamaria, R., Di Rosa, M., and Izzo, A. A. (2004). Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. *J. Neurochem.* 89, 134–141. doi: 10.1111/j.1471-4159.2003.02327.x

Janefjord, E., Mååg, J. L., Harvey, B. S., and Smid, S. D. (2013). Cannabinoid effects on β amyloid fibril and aggregate formation, neuronal and microglial-activated neurotoxicity in vitro. *Cell. Mol. Neurobiol.* 34, 31–42. doi: 10.1007/s10571-013-9984-x

Jung, K. M., Astarita, G., Yasar, S., Vasilevko, V., Cribbs, D. H., Head, E., et al. (2012). An amyloid β42-dependent deficit in anandamide mobilization is associated with cognitive dysfunction in Alzheimer's disease. *Neurobiol. Aging* 33, 1522–1532. doi: 10.1016/j.neurobiolaging.2011.03.012

Keller, J. N., Hanni, K. B., and Markesbery, W. R. (2000). Impaired proteasome function in Alzheimer's disease. *J. Neurochem.* 75, 436–439. doi: 10.1046/j.1471-4159.2000.0750436.x

Khaspekov, L. G., Brenz-Verca, M. S., Frumkina, L. E., Hermann, H., Marsicano, G., and Lutz, B. (2004). Involvement of brain-derived neurotrophic factor in cannabinoid receptor-dependent protection against excitotoxicity. *Eur. J. Neurosci.* 19, 1691–1698. doi: 10.1111/j.1460-9568.2004.03285.x

Koppel, J., Bradshaw, H., Goldberg, T. E., Khalili, H., Marambaud, P., Walker, M. J., et al. (2009). Endocannabinoids in Alzheimer's disease and their impact on normative cognitive performance: a case–control and cohort study. *Lipids Health Dis.* 8, 2. doi: 10.1186/1476-511X-8-2

Kovacs, G. G., Alafuzoff, I., Al-Sarraj, S., Arzberger, T., Bogdanovic, N., Capellari, S., et al. (2008). Mixed brain pathologies in dementia: the BrainNet Europe Consortium experience. *Dement. Geriatr. Cogn. Disord.* 26, 343–350. doi: 10.1159/000161560

Krishnan, S., Cairns, R., and Howard, R. (2009). Cannabinoids for the treatment of dementia. *Cochrane Database Syst. Rev.* 2:CD007204. doi: 10.1002/14651858.CD007204.pub2

Lazarov, O., and Marr, R. A. (2010). Neurogenesis and Alzheimer's disease: at the crossroads. *Exp. Neurol.* 223, 267–281. doi: 10.1016/j.expneurol.2009.08.009

Lee, J., Fukumoto, H., Orne, J., Klucken, J., Raju, S., Vanderburg, C. R., et al. (2005). Decreased levels of BDNF protein in Alzheimer temporal cortex are independent of BDNF polymorphisms. *Exp. Neurol.* 194, 91–96. doi: 10.1016/j.expneurol.2005.01.026

Lee, J. H., Agacinski, G., Williams, J. H., Wilcock, G. K., Esiri, M. M., Francis, P. T., et al. (2010). Intact cannabinoid CB1 receptors in the Alzheimer's disease cortex. *Neurochem. Int.* 57, 985–989. doi: 10.1016/j.neuint.2010.10.010

Maccarrone, M., Dainese, E., and Oddi, S. (2010). Intracellular trafficking of anandamide: new concepts for signaling. *Trends Biochem. Sci.* 35, 601–608. doi: 10.1016/j.tibs.2010.05.008

Mackie, K., and Hille, B. (1992). Cannabinoids inhibit N-type calcium channels in neuroblastoma–glioma cells. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3825–3829. doi: 10.1073/pnas.89.9.3825

Maldonado, R., Berrendero, F., Ozaita, A., and Robledo, P. (2011). Neurochemical basis of cannabis addiction. *Neuroscience* 181, 1–17. doi: 10.1016/j.neuroscience.2011.02.035

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208

Martín, V., Fabelo, N., Santpere, G., Puig, B., Marín, R., Ferrer, I., et al. (2010). Lipid alterations in lipid rafts from Alzheimer's disease human brain cortex. *J. Alzheimers Dis.* 19, 489–502. doi: 10.3233/JAD-2010-1242

Martín-Moreno, A. M., Brera, B., Spuch, C., Carro, E., García-García, L., Delgado, M., et al. (2012). Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *J. Neuroinflammation* 9, 8. doi: 10.1186/1742-2094-9-8

Martín-Moreno, A. M., Reigada, D., Ramírez, B. G., Mechoulam, R., Innamorato, N., Cuadrado, A., et al. (2011). Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. *Mol. Pharmacol.* 79, 964–973. doi: 10.1124/mol.111.071290

Milton, N. G. (2002). Anandamide and noladin ether prevent neurotoxicity of the human amyloid-beta peptide. *Neurosci. Lett.* 332, 127–130. doi: 10.1016/S0304-3940(02)00936-9

Morozov, Y. M., Dominguez, M. H., Varela, L., Shanabrough, M., Koch, M., Horvath, T. L., et al. (2013). Antibodies to cannabinoid type 1 receptor co-react with stomatin-like protein 2 in mouse brain mitochondria. *Eur. J. Neurosci.* 38, 2341–2348. doi: 10.1111/ejn.12237

Mulder, J., Zilberter, M., Pasquare, S. J., Alpar, A., Schulte, G., Ferreira, S. G., et al. (2011). Molecular reorganization of endocannabinoid signalling in Alzheimer's disease. *Brain* 134, 1041–1060. doi: 10.1093/brain/awr046

Nadler, V., Mechoulam, R., and Sokolovsky, M. (1993). The non-psychotropic cannabinoid (+)-(3S,4S)-7-hydroxy-delta 6-tetrahydrocannabinol 1,1-dimethylheptyl (HU-211) attenuates *N*-methyl-D-aspartate receptor-mediated neurotoxicity in primary cultures of rat forebrain. *Neurosci. Lett.* 162, 43–45. doi: 10.1016/0304-3940(93)90555-Y

Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A., et al. (2005). Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J. Neuropathol. Exp. Neurol.* 64, 113–122.

Nomura, D. K., Morrison, B. E., Blankman, J. L., Long, J. Z., Kinsey, S. G., Marcondes, M. C., et al. (2011). Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. *Science* 334, 809–813. doi: 10.1126/science.1209200

Noonan, J., Tanveer, R., Klompas, A., Gowran, A., McKiernan, J., and Campbell, V. A. (2010). Endocannabinoids prevent β-amyloid-mediated lysosomal destabilization in cultured neurons. *J. Biol. Chem.* 285, 38543–38554. doi: 10.1074/jbc.M110.162040

Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Meozzi, P. A., Myers, L., et al. (2008). Functional expression of brain neuronal CB2 cannabinoid receptors are involved in the effects of drugs of abuse and in depression. *Ann. N.Y. Acad. Sci.* 1139, 434–449. doi: 10.1196/annals.1432.036

Pacher, P., Bátkai, S., and Kunos, G. (2005). Cardiovascular pharmacology of cannabinoids. *Handb. Exp. Pharmacol.* 168, 599–625. doi: 10.1007/3-540-26573-2_20

Pamplona, R., Dalfó, E., Ayala, V., Bellmunt, M. J., Prat, J., Ferrer, I., et al. (2005). Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation: effects of Alzheimer disease and identification of lipoxidation targets. *J. Biol. Chem.* 280, 21522–21530. doi: 10.1074/jbc.M502255200

Passmore, M. J. (2008). The cannabinoid receptor agonist nabilone for the treatment of dementia-related agitation. *Int. J. Geriatr. Psychiatry* 23, 116–117. doi: 10.1002/gps.1828

Peng, S., Garzon, D. J., Marchese, M., Klein, W., Ginsberg, S. D., Francis, B. M., et al. (2009). Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease. *J. Neurosci.* 29, 9321–9329. doi: 10.1523/JNEUROSCI.4736-08.2009

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *Br. J. Pharmacol.* 153, 199–215. doi: 10.1038/sj.bjp.0707442

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. *Pharmacol. Rev.* 62, 588–631. doi: 10.1124/pr.110.003004

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* 4, 873–884. doi: 10.1038/nrn1247

Piro, J. R., Benjamin, D. I., Duerr, J. M., Pi, Y., Gonzales, C., Wood, K. M., et al. (2012). A dysregulated endocannabinoid-eicosanoid network supports pathogenesis in a mouse model of Alzheimer's disease. *Cell Rep.* 1, 617–623. doi: 10.1016/j.celrep.2012.05.001

Ramírez, B. G., Blázquez, C., Gómez del Pulgar, T., Guzmán M., and de Ceballos, M. L. (2005). Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J. Neurosci.* 25, 1904–1913. doi: 10.1523/JNEUROSCI.4540-04.2005

Ruiz-Valdepeñas, L., Benito, C., Tolón, R. M., Martínez-Orgado, J. A., and Romero, J. (2010). The endocannabinoid system and amyloid-related diseases. *Exp. Neurol.* 224, 66–73. doi: 10.1016/j.expneurol.2010.03.024

Sardi, F., Fassina, L., Venturini, L., Inquuscio, M., Guerriero, F., Rolfo, E., et al. (2011). Alzheimer's disease, autoimmunity and inflammation. The good, the bad and the ugly. *Autoimmun. Rev.* 11, 149–153. doi: 10.1016/j.autrev.2011.09.005

Scharfman, H., Goodman, J., Macleod, A., Phani, S., Antonelli, C., and Croll, S. (2005). Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Exp. Neurol.* 192, 348–356. doi: 10.1016/j.expneurol.2004.11.016

Selkoe, D. J. (2012). Preventing Alzheimer's disease. *Science* 337, 1488–1492. doi: 10.1126/science.1228541

Silvestri, C., and Di Marzo, V. (2013). The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell Metab.* 17, 475–490. doi: 10.1016/j.cmet.2013.03.001

Simic, G., Stanic, G., Mladinov, M., Jovanov-Milosevic, N., Kostovic, I., and Hof, P. R. (2009). Does Alzheimer's disease begin in the brainstem? *Neuropathol. Appl. Neurobiol.* 35, 532–554. doi: 10.1111/j.1365-2990.2009.01038.x

Solas, M., Francis, P. T., Franco, R., and Ramírez, M. J. (2013). CB2 receptor and amyloid pathology in frontal cortex of Alzheimer's disease patients. *Neurobiol. Aging* 34, 805–808. doi: 10.1016/j.neurobiolaging.2012.06.005

Stella, N., Schweitzer, P., and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388, 773–778. doi: 10.1038/42015

Stumm, C., Hiebel, C., Hanstein, R., Purrio, M., Nagel, H., Conrad, A., et al. (2013). Cannabinoid receptor 1 deficiency in a mouse model of Alzheimer's disease leads to enhanced cognitive impairment despite of a reduction in amyloid deposition. *Neurobiol. Aging* 34, 2574–2584. doi: 10.1016/j.neurobiolaging.2013.05.027

Sultana, R., and Butterfield, D. A. (2010). Role of oxidative stress in the progression of Alzheimer's disease. *J. Alzheimers Dis.* 19, 341–353. doi: 10.3233/JAD-2010-1222

Tanveer, R., Gowran, A., Noonan, J., Keating, S. E., Bowie, A. G., and Campbell, V. A. (2012). The endocannabinoid, anandamide, augments Notch-1 signaling in cultured cortical neurons exposed to amyloid-β and in the cortex of aged rats. *J. Biol. Chem.* 287, 34709–34721. doi: 10.1074/jbc.M112.350678

Tolón, R. M., Núñez, E., Pazos, M. R., Benito, C., Castillo, A. I., Martínez-Orgado, J. A., et al. (2009). The activation of cannabinoid CB2 receptors stimulates in situ and in vitro beta-amyloid removal by human macrophages. *Brain Res.* 1283, 148–154. doi: 10.1016/j.brainres.2009.05.098

Twitchell, W., Brown, S., and Mackie, K. (1997). Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J. Neurophysiol.* 78, 43–50.

van der Stelt, M., Mazzola, C., Esposito, G., Matias, I., Petrosino, S., De Filippis, D., et al. (2006). Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. *Cell. Mol. Life Sci.* 63, 1410–1424. doi: 10.1007/s00018-006-6037-3

Van Sickle, M. D., Duncan, M., Kingsley, P. J., Mouihate, A., Urbani, P., Mackie, K., et al. (2005). Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 310, 329–332. doi: 10.1126/science.1115740

Viscomi, M. T., Oddi, S., Latini, L., Pasquariello, N., Florenzano, F., Bernardi, G., et al. (2009). Selective CB2 receptor agonism protects central neurons from remote axotomy-induced apoptosis through the PI3K/Akt pathway. *J. Neurosci.* 29, 4564–4570. doi: 10.1523/JNEUROSCI.0786-09.2009

Volicer, L., Stelly, M., Morris, J., McLaughlin, J., and Volicer, B. J. (1997). Effects of dronabinol on anorexia and disturbed behavior in patients with Alzheimer's disease. *Int. J. Geriatr. Psychiatry* 12, 913–919. doi: 10.1002/(SICI)1099-1166(199709)12:9<913::AID-GPS663>3.0.CO;2-D

Wagner, J. A., Járai, Z., Bátkai, S., and Kunos, G. (2001). Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB(1) receptors. *Eur. J. Pharmacol.* 423, 203–210. doi: 10.1016/S0014-2999(01)01112-8

Walther, S., Mahlberg, R., Eichmann, U., and Kunz, D. (2006). Delta-9-tetrahydrocannabinol for nighttime agitation in severe dementia. *Psychopharmacology (Berl.)* 185, 524–528. doi: 10.1007/s00213-006-0343-1

Walther, S., Schüpbach, B., Seifritz, E., Homan, P., and Strik, W. (2011). Randomized, controlled crossover trial of dronabinol, 2.5 mg, for agitation in 2 patients with dementia. *J. Clin. Psychopharmacol.* 31, 256–258. doi: 10.1097/JCP.0b013e31820e861c

Westlake, T. M., Howlett, A. C., Bonner, T. I., Matsuda, L. A., and Herkenham, M. (1994). Cannabinoid receptor binding and messenger RNA expression in human brain: an in vitro receptor autoradiography and in situ hybridization histochemistry study of normal aged and Alzheimer's brains. *Neuroscience* 63, 637–652. doi: 10.1016/0306-4522(94)90511-8

Wilson, R. I., and Nicoll, R. A. (2002). Endocannabinoid signaling in the brain. *Science* 296, 678–682. doi: 10.1126/science.1063545

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., and Naguib, M. (2013). Activation of the CB2 receptor system reverses amyloid-induced memory deficiency. *Neurobiol. Aging* 34, 791–804. doi: 10.1016/j.neurobiolaging.2012.06.011

Zhuang, S. Y., Bridges, D., Grigorenko, E., McCloud, S., Boon, A., Hampson, R. E., et al. (2005). Cannabinoids produce neuroprotection by reducing intracellular calcium release from ryanodine-sensitive stores. *Neuropharmacology* 48, 1086–1096. doi: 10.1016/j.neuropharm.2005.01.005

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Prevention approaches in a preclinical canine model of Alzheimer's disease: benefits and challenges

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Aged dogs spontaneously develop many features of human aging and Alzheimer's disease (AD) including cognitive decline and neuropathology. In this review, we discuss age-dependent learning tasks, memory tasks, and functional measures that can be used in aged dogs for sensitive treatment outcome measures. Neuropathology that is linked to cognitive decline is described along with examples of treatment studies that show reduced neuropathology in aging dogs (dietary manipulations, behavioral enrichment, immunotherapy, and statins). Studies in canine show that multi-targeted approaches may be more beneficial than single pathway manipulations (e.g., antioxidants combined with behavioral enrichment). Aging canine studies show good predictive validity for human clinical trials outcomes (e.g., immunotherapy) and several interventions tested in dogs strongly support a prevention approach (e.g., immunotherapy and statins). Further, dogs are ideally suited for prevention studies as they the age because onset of cognitive decline and neuropathology strongly support longitudinal interventions that can be completed within a 3–5 year period. Disadvantages to using the canine model are that they lengthy, use labor-intensive comprehensive cognitive testing, and involve costly housing (almost as high as that of non-human primates). However, overall, using the dog as a preclinical model for testing preventive approaches for AD may complement work in rodents and non-human primates.

Keywords: antioxidant diet, atorvastatin, behavioral enrichment, beta-amyloid, combination treatment, dog, immunotherapy, statin

INTRODUCTION

Alzheimer's disease (AD) is a progressive dementia associated with the accumulation of beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFT; McKhann et al., 1984; Mirra, 1997). Currently five drugs are approved for use by the FDA to manage the symptoms of AD, although none target disease pathways and all may provide only symptomatic relief. These drugs include donepezil, rivastigmine, tacrine, galantamine, and memantine (Aisen et al., 2012). However, these five approved drugs target only two pathways, one involving acetylcholinesterase inhibition and the second is an NMDA receptor antagonist. Thus, preclinical studies are critical for developing and testing new disease-modifying interventions that can be taken to clinical trials in patients with AD. Typically, studies in rodents are the earliest steps in this process to screen drugs that target AD pathways with most preclinical studies of AD interventions using transgenic mouse models of AD. Subsequently, safety studies in humans are followed by a clinical trial in AD patients. Many of the AD clinical trials currently underway target different pathogenic pathways active in the disease[1].

Several clinical trials are targeting the reduction of Aβ. The rationale stems from predictions based on the amyloid hypothesis, originally proposed by Hardy and Higgins (1992) and updated by Hardy (2006) suggesting that Aβ is a critical causative factor in the disease. Thus, the focus of several clinical trials has been to either reduce production (secretase inhibition) or increase clearance (immunotherapy) of Aβ. Unfortunately, most of these promising new approaches have failed in clinical trials (for review, see Mullane and Williams, 2013). Possible reasons for failure include but are not limited to: (1) the targets are not critical for AD pathogenesis and dementia, (2) the single pathway reductionist approach may be insufficient, (3) the treatment is too late (suggesting prevention studies) or, (4) the preclinical animal model was not a predictor of human clinical trials outcomes. In this review we first discuss the canine model of human aging and AD, how dogs are well suited for prevention studies based on established sensitive cognitive tasks and brain pathology measures, and then outcomes of preclinical studies with both single and multiple targets that may predict human clinical trial outcomes.

THE CANINE MODEL OF HUMAN AGING AND ALZHEIMER'S DISEASE

Some of the most commonly studied animal models of human brain aging are rodents and non-human primates (Gallagher and Rapp, 1997). Other animals, including wolves, bears, cats, and dogs, naturally develop human-like neuropathology (Head et al., 2001). Of these animals, cats and dogs tend to have similar

[1]http://clinicaltrials.gov/ct2/results%3Fterm=alzheimer%27s%26pg=6

living environments to humans (Head et al., 2001). Canines, however, show cognitive decline with age and develop most aspects of neuropathology seen in aged human brain including AD patients (Cummings et al., 1996b; Cotman and Head, 2008). Such neuropathology includes Aβ pathology, reduced brain volume, neuronal loss, and impaired neurogenesis (Head, 2001; Cotman and Head, 2008). In addition to the similar cognitive decline and accumulation of neuropathological hallmarks to humans with AD, drugs exhibit similar pharmacokinetics when administered to dogs or humans [for example statins – (Gerson et al., 1989; Alberts, 1990)], making them an appropriate model for translational studies on therapeutic drugs. Not only are dogs easy to handle due to their long history of domestication, but pet dogs also share similar living conditions and diets to humans (Cummings et al., 1996b; Parker et al., 2004; Axelsson et al., 2013). Canines are highly motivated by food reward when conducting cognitive tests, which makes them cooperative research subjects by reducing or eliminating deprivation protocols for motivation. Thus, this cooperativeness eliminates many physiological stressors that can affect cognitive testing results present in other animal models such as rodents that require food deprivation or cold water for motivation (Blizard et al., 2003). The similar cognitive decline and accumulation of neuropathology to humans makes the canine model of aging useful for translational research on neurodegenerative diseases, especially AD.

COGNITIVE OUTCOME MEASURES FOR PREVENTION STUDIES IN AGING DOGS

We describe several measures of cognition that are age-sensitive and treatment-sensitive in dogs that can be used as intervention outcome measures to assess different cognitive abilities with analogous tasks in non-human primates and in humans (**Table 1**). Much like humans, the aging canine shows cognitive decline with various cognitive domains and cortical pathways being differentially affected (Milgram et al., 1994). Dogs show cognitive deficits due to age in tests measuring complex learning, executive function, spatial learning and attention, and memory (Milgram et al., 1994, 2002b; Head et al., 1998a; Cotman et al., 2002; Tapp et al., 2003a,b, 2004b; Christie et al., 2005; Siwak et al., 2005; Studzinski et al., 2006). In addition to cognitive domain variability, individual dogs also show variability in cognitive function as seen in humans (Adams et al., 2000). This variability becomes most apparent in old canines, and using spatial learning and memory tasks, we are able to distinguish three groups of variability: (1) successful agers, (2) impaired dogs whose scores fell two SD above the mean of the young animals, and (3) severely impaired dogs who failed to learn the task (Head et al., 2001). The availability of age-matched animals with and without cognitive deficits allowed researchers to determine which types of neuropathology contribute to individual cognitive impairments in these animals (e.g., Head et al., 1998a).

Several tasks, similar to those used for testing cognition in non-human primates, have been developed to measure cognitive decline in the aging canine (Milgram et al., 1994, 1999, 2002a; **Table 1**). Such tasks include landmark discrimination, oddity discrimination, object, size and black/white discrimination and reversal tasks, and a spatial memory task. In our laboratory studies using these cognitive tasks, all testing occurs in a modified Wisconsin General Testing Apparatus such that the motor and sensory demands are consistent across tasks (Milgram et al., 1994). For each task, 10–12 trials are given per day and dogs are tested daily until a predetermined criterion level of performance is reached; total error scores are added up across days to provide a measure of learning and/or memory for each animal. These tasks are described in more detail below to illustrate how a test battery can be developed to measure the function of several brain circuits that may be differentially affected by age and/or treatment in aging dogs.

The *landmark discrimination task*, which measures visuospatial function and allocentric learning, involves presenting dogs with two identical objects, one of which is adjacent to a third object that serves as a landmark (Milgram et al., 1999). Animals are required to recognize that the landmark is an indicator of which object covers the food reward, and selection of the object closest to this landmark by the animal is considered a correct response. The task is made successively more difficult by placing the landmark further away from the object covering the reward. Previous work shows that aged dogs are impaired on the landmark task and show age decrements in their ability to determine how close the landmark is to the correct object (Milgram et al., 1999, 2002a).

The *oddity discrimination task* measures complex learning, as well as prefrontal cortex function (Cotman et al., 2002). Aged dogs show deficits in oddity discrimination learning (Cotman et al., 2002; Milgram et al., 2002b). In this task, dogs are presented with three objects simultaneously, two of which are identical and a third that is unique. A correct response is indicated when the dog chooses the unique object, resulting in a reward. To prevent a floor effect and detect progressive age decline, the oddity aspect of this task is made successively more difficult. Animals progress through four sets of three objects and each subsequent set contains a unique object, which is more difficult to distinguish from others than the previous set (Milgram et al., 2002b). Interestingly, young dogs can solve this problem by using the strategy of selecting the novel object for each successive set of objects such that error scores plateau; in contrast, aged dogs do not learn a strategy but re-learn each set of objects as a new problem (Cotman et al., 2002; Milgram et al., 2002b).

Tests of *object, size and black/white discrimination* are administered to measure associative learning ability. Object discrimination involves presenting dogs with two different objects simultaneously with one of the two objects consistently rewarded. Dogs must learn to select the same object each presentation with the left/right position being randomly determined. Similarly, the size discrimination objects differ in size (small/large) and the black/white discrimination task objects differ only in color (black/white; Milgram et al., 2005). Object, size and black/white discrimination are also progressively more difficult for animals to solve given the similarities in the objects increasing. Thus, these three tasks in combination can serve as different test versions (much like in clinical studies in people) to assess longitudinal changes in learning while reducing practice effects (Milgram et al., 2005).

Executive function can be evaluated immediately after discrimination learning has been completed by using the *object, size or*

Table 1 | Cognitive domains assessed in dog aging and comparison with non-human primate tasks and analogous tasks used in human neuropsychological testing.

Cognitive domain	Dog task	Localization in dog brain	Non-human primate tasks	Examples of human neuropsychological tasks**
Learning	Visual discrimination learning	Medial temporal lobe/parietal lobe*	Visual discrimination learning (Rapp, 1990; Lai et al., 1995)	Digit copy, rotary pursuit, face discrimination (Cronin-Golomb, 2001), object discrimination (Freedman and Oscar-Berman, 1989; Boutet et al., 2007)
Memory	Spatial delayed non-match to sample acquisition	Dorsolateral prefrontal cortex (Christie et al., 2005)	Delayed response task (Arnsten and Goldman-Rakic, 1985; Walker et al., 1988)	Delayed recognition and recall, digit span (Lezak et al., 2004)
	Spatial delayed non-match to sample memory	Hippocampus (Kowalska, 1995)	Delayed response task (Arnsten and Goldman-Rakic, 1985; Walker et al., 1988)	
Executive function	Visual reversal learning	Prefrontal cortex/medial temporal lobe (Warren, 1964)	Visual reversal learning (Rapp, 1990; Lai et al., 1995)	Card or object sorting tasks, set shifting, response inhibition (Kramer and Quitania, 2007)
	Oddity discrimination	Prefrontal cortex/medial temporal lobe*	N/A	
Visuospatial function	Landmark discrimination	Prefrontal cortex/parietal cortex*	Landmark discrimination (Pohl, 1973)	Visual construction, block design, spatial learning (Freedman and Oscar-Berman, 1989; Boutet et al., 2007)
	Egocentric spatial learning	Hippocampus/medial temporal lobe*	Spatial learning (Lai et al., 1995)	

**Proposed localization – not confirmed in lesion studies in dogs.*
***Neuropsychological tasks for humans that assess function in similar cognitive domains.*
Modified from Table 1 in Martin et al. (2011b).

black/white reversal objects. The reversal tasks differ from the original discrimination task in that the positive and negative objects for reward contingencies are reversed after animals have learned the initial discrimination (Milgram et al., 2004, 2005). Reversing the reward contingencies can show perseverative behaviors (persistent choice of previously correct object), which are frontal cortex dependent (Warren, 1964). A subset of the discrimination learning tasks and all reversal learning tasks are age-dependent, with reversal learning being consistently more impaired with age (Milgram et al., 1994, 2004, 2005; Tapp et al., 2004b; Siwak et al., 2005).

Memory also declines with age in dogs. The most useful age-sensitive task we have used is a *spatial memory task*, in which dogs are required to recognize the location of a sample stimulus and then respond to a different location during the test trial. We refer to this as a delayed non-match to position task (DNMP) and it involves showing animals a single object covering a food reward either on the left or right food well. After animals move the object and obtain the reward, the object is withdrawn from sight for a predetermined delay period (e.g., 10 s). Subsequently animals are given two identical objects to choose from; one is the same object in the same position as before and one is in a novel position. The correct response is to select the object covering the novel location. Results published in 1995, Head et al. (1995) suggested that the task was age-sensitive. We subsequently developed a three-choice visuospatial working memory task that allows determination of the differential age-dependent strategies (e.g., cognitive or stimulus-dependent strategies) dogs use in solving the problem (Chan et al., 2002). In this task, rather than just the left and right food wells are used but a center well is also included to make the task more difficult. Further, this task shows minimal practice effects in longitudinal studies (Head et al., 2008). We identified the time course of the development of cognitive decline and found that deterioration in spatial ability occurs early in the aging process, between 6 and 7 years of age in dogs (Studzinski et al., 2006).

BEHAVIORAL/FUNCTIONAL OUTCOME MEASURES FOR PREVENTION STUDIES IN DOGS

In addition to cognitive outcome measures, researchers and veterinarians are interested in measuring functional outcomes. Further, laboratory-based cognitive testing as described above is labor intensive and requires many months to years to obtain data. An *open field test* can be used to observe the behavioral patterns of animals in an empty room for 10 min. During this task, movement, sniffing, urinating, grooming, rearing, jumping, vocalization, and inactivity are noted (Head and Milgram, 1992; Siwak et al., 2000, 2001). *Self-recognition* can be evaluated through the mirror test, originally developed for primates (Gallup, 1968; de Veer et al., 2003), by observing the reaction of each animal with a mirror and their reflection. *Exploratory behavior* of canines can be assessed through a curiosity test in which animals are presented with various novel play objects. During their time with the objects, the amount of time the dogs spend in physical contact with or sitting next to the objects is recorded as well as their frequency of sniffing the objects (Siwak et al., 2001). Social responsiveness of dogs can be gaged through a few different tasks: a human interaction test, silhouette test, and the model dog test. A *human interaction* test is performed by the presence of a person in the middle of the room and recording the reaction of the dog to that person by measuring the time the dog is in physical contact with the person, time sitting or standing beside the person, and frequency sniffing the person (Head et al., 1997). The *silhouette test* records the animals frequency of sniffing the front and rear regions of a cardboard silhouette of a dog posted onto a wall (Fox and Weisman, 1970). The model dog test also records the sniffing frequency of the dogs, but this time in response to the presence of a life size model dog in the center of a room (Siwak et al., 2001).

Behavioral patterns in these tasks show age effects as well as differential effects based on the presence of intact/impaired cognition. Siwak et al. (2001) characterized the behavioral profiles of young (2–4 years), aged (9–15 years) cognitively impaired, and aged non-impaired beagles. Young dogs tend to show greater responsiveness to changes in environments such as the addition of novel objects and a person. They also showed greater social responsiveness spending the most time next to or sniffing a person, silhouette, and model dog. Aged unimpaired dogs were still responsive to alterations in environment, but to a lesser degree than the young animals. Additionally, aged unimpaired dogs spent the least amount of time reacting to the mirror during the self-recognition task. Unlike either the young or aged unimpaired canines, the aged impaired canines were unresponsive to all stimuli presented to the environment and randomly moved about the room in pacing/aimless behavior. However, the aged impaired dogs did spend the most time interacting with the mirror in the self-recognition test (Siwak et al., 2001).

Measures of canine function can also be assessed in a clinical setting (Landsberg and Ruehl, 1997; Landsberg and Araujo, 2005; Landsberg et al., 2012). Clinical measures have been developed consisting of pet *dog owner based evaluation* of dog behavioral changes (Colle et al., 2000; Pugliese et al., 2006a, 2007; Bosch et al., 2012, 2013; Landsberg et al., 2012) similar to those used in human clinical evaluations, such as the mini mental state exam (MMSE). Although there are different versions of these questionnaires, all appear to be sensitive to the presence of canine cognitive dysfunction (Landsberg et al., 2012). The evaluation consists of items such as walking, posture/emotion of expression, elimination behavior, life rhythm, play behavior, exploratory behavior, learned specific behavior, adaptive capabilities, and interactions with other animals or with owners. The items of individual questionnaires can be used to derive scores that distinguish between normally and pathologically aging dogs. Adult and older dogs generally score worse with these types of evaluation tools, and old dogs show individual variability in terms of the amount of cognitive dysfunction reported (Bosch et al., 2012).

DOG NEUROPATHOLOGY AND OUTCOME MEASURES FOR PREVENTION STUDIES

Just as canines can exhibit cognitive decline with age similar to aging humans and patients with AD, several human-type neuropathologies have been reported in dogs (Cotman and Head, 2008). In particular, the canine model has long been suggested as an excellent model of Aβ pathogenesis (Wisniewski et al., 1990).

Several changes observed in the aged canine brain are associated with cognition and are discussed below.

Individuals with AD show significant cortical and hippocampal atrophy relative to non-demented age matched controls (Alavi et al., 1993; Raz et al., 1998) and losses *in brain volume* correlate with cognitive decline (Ezekiel et al., 2004; Du et al., 2005). Similar events are seen in aged canines. On cross sectional MR imaging, aging canines show increased cortical atrophy and ventricular widening (Su et al., 1998; Gonzalez-Soriano et al., 2001; Kimotsuki et al., 2005). Ventricular widening over time was observed by MRI in a 3-year longitudinal study (Su et al., 2005). Canine cortical atrophy occurs earliest in the prefrontal cortex and later with age in the hippocampus (Tapp et al., 2004a). As with humans, the more extensive the cortical/hippocampal atrophy seen in aged canines the more pronounced the cognitive deficits (Tapp et al., 2004a; Rofina et al., 2006).

Neuronal loss occurs in human brain aging and could explain the brain volume losses seen in brain imaging (West, 1993; Simic et al., 1997). With normal brain aging, neuronal loss is only seen in the hilus (West, 1993; West et al., 1994), while neuronal loss is much more widespread in individuals with AD (Bobinski et al., 1997; West et al., 2000). Individuals with AD experience neuronal loss in the CA1, CA2, CA4, and subiculum of the hippocampus (Bobinski et al., 1997; West et al., 2000; Price et al., 2001). In aged beagles, the hilus of the dentate gyrus showed fewer neurons compared to younger dogs (Siwak-Tapp et al., 2008). Beagles with fewer neurons in the hilus made significantly more errors when performing the size discrimination task (Siwak-Tapp et al., 2008). Similarly, Pugliese et al. (2007) found that a loss of Purkinje cells in canines correlated with data acquired by questionnaires quantifying behavioral deficits. However, neuronal loss may not account for all of the brain atrophy observed by MR as the loss of neuronal dendritic spines occurs with AD (Knobloch and Mansuy, 2008; Overk and Masliah, 2014) but to our knowledge, there are currently no studies published evaluating similar changes with age in dogs.

While selective neuronal loss may occur with aging, the brain is also able to produce new neurons. The hippocampus, for example, grows new neurons in the subgranular layer (Eriksson et al., 1998). *Neurogenesis* has been explored in aged beagles using BrdU and doublecortin protein staining methods. Siwak-Tapp et al. (2007) measured neurogenesis in aged beagles using BrdU and found that animals over the age of 13 experienced a significant loss of neurogenesis. Fewer newer BrdU positive neurons was associated with poorer cognitive function in learning and memory and learning ability (Siwak-Tapp et al., 2007).

Neuronal dysfunction could result in abnormal production of critical *neurotransmitters* in the brain. Thus, one potential target for therapeutics in AD is to manipulate or restore decreased neurotransmitter levels. Some drugs targeting neurotransmitters are already available as treatments for AD; however, as mentioned earlier; these drugs at best provide only symptomatic relief. Neurotransmitter deficits have not been thoroughly explored in canines. In humans, decreases in specific neurotransmitter systems are associated with aging and AD (Meltzer et al., 1998; Ballard et al., 2005; Schliebs and Arendt, 2006; Rissman et al., 2007). Dogs with Aβ accumulation in the gyrus proreus possess fewer serotonergic neurons (Bernedo et al., 2009). A decrease in receptor binding of serotonin is seen with age in dogs over 8 years of age (Peremans et al., 2002). Animals with high levels of Aβ in the prefrontal cortex experience a loss of noradrenergic neurons in the locus ceruleus, which is also associated with cognitive dysfunction (Insua et al., 2010). Acetylcholinesterase density is reduced in granule cells of the cerebellum with age (Pugliese et al., 2007). Aged canines experience a loss of gamma-aminobutyric acid interneurons in the prefrontal cortex (Pugliese et al., 2004), as well as the CA1 and dentate gyrus of the hippocampus (Hwang et al., 2008b). Additionally, a loss of glutamic acid decarboxylase 67 neurons in CA1 of the hippocampus is seen in aged canines over 10 years of age (Hwang et al., 2008b). Thus, similar patterns of age-associated neurotransmitter system dysfunction appear in aging dogs and may be a suitable model system in which to develop or test novel neurotransmitter pathway-based interventions. The pathogenic mechanisms underlying neuronal dysfunction, neurotransmitter losses and death may include, e.g., the deposition of Aβ, cerebrovascular dysfunction, or oxidative damage.

Beta-amyloid (*A*β) is derived from a longer precursor protein, the amyloid precursor protein (APP). The APP sequence of *Canis familiaris* has 98% homology with human APP[2] and an identical amino acid sequence (Selkoe et al., 1987; Johnstone et al., 1991). Additionally, dog Aβ peptides may undergo the same posttranslational modifications as in humans (Satou et al., 1997; Azizeh et al., 2000). These similarities make canines a viable aging model without the need for genetic modification or overexpression of mutant human proteins (Selkoe et al., 1987).

The Aβ present in canines is ultrastructurally fibrillar and, though more compact deposits may form, it generally aggregates into diffuse plaques (Giaccone et al., 1990; Russell et al., 1992; Uchida et al., 1992; Cummings et al., 1993; Morys et al., 1994; Torp et al., 2000a,b). This type of Aβ deposition most resembles early AD pathology (Morris et al., 1996; Markesbery et al., 2006; Cotman and Head, 2008; **Figures 1A,B**). Since most AD therapeutics studied today are likely to have a greater affect if applied earlier in the disease progression, the early AD-like pathology canines produce makes them an attractive model for prevention studies (Martin et al., 2011b). As with cognitive decline, AD-like neuropathology has a region specific progression in both humans and canines (Wisniewski et al., 1970; Selkoe et al., 1987; Giaccone et al., 1990; Braak and Braak, 1991; Head et al., 2000; Thal et al., 2002). Though this progression in dogs is similar to that reported in humans, it is not identical. In canines, the accumulation of Aβ begins in the prefrontal cortex (approximately 8 years at age of onset) and continues to develop with increasing age to include other regions such as the temporal and occipital cortex (Russell et al., 1996; Head et al., 2000; Cotman and Head, 2008). The severity of neuropathology can vary between individual animals but can be linked to the extent of cognitive decline (Cummings et al., 1996a; Head et al., 1998b; Colle et al., 2000; Rofina et al., 2006). For instance, animals who perform worse in reversal learning tasks have greater Aβ pathology in the prefrontal cortex, while those deficient in size discrimination learning show higher amounts of Aβ in the entorhinal

[2]http://www.ensembl.org/Canis_familiaris/

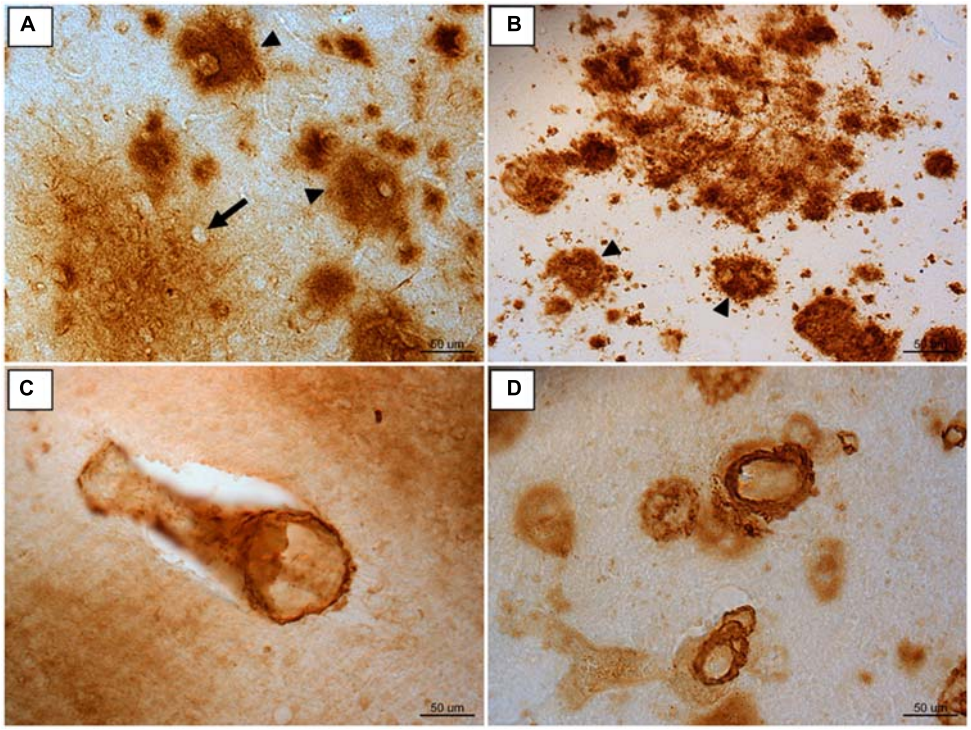

FIGURE 1 | Immunoreactivity for Aβ 1-42 in frontal cortex brain tissue of (A) an aged canine and (B) an aged human. Compact Aβ deposits are similar in humans and canines (arrow head). The outline of an intact neuron enveloped by a diffuse plaque is visible (arrow). Aβ 1-40 immunoreactivity of cerebral amyloid angiopathy is similar in aged canine occipital cortex **(C)** and aged human occipital cortex **(D)**. Reproduced from Martin et al. (2011b).

cortex (Cummings et al., 1996a; Head et al., 1998a; Pop et al., 2010b).

Aβ peptide can also be measured in the cerebrospinal fluid (CSF) of dogs (Sarasa et al., 2013). Measuring CSF Aβ as a ratio of Aβ 42/Aβ 40 is a good predictor of Aβ in the brain in dogs (Head et al., 2010). While brain Aβ increases with age, CSF Aβ decreases with age reflecting the hypothesis that Aβ migrates from the periphery and deposits in the brain with age and AD.

Aside from the fibrillar Aβ found in diffuse plaques in AD, a smaller, more soluble form of Aβ – oligomeric Aβ – is also seen in the aged dog brain. This more toxic form of Aβ affects synaptic function and can even be found in plaques (Walsh et al., 2002; Kayed et al., 2003; Selkoe, 2008). Higher levels of oligomers are present in canines and humans with increasing age and cognitive decline. The greater the cognitive deficit, the more prevalent oligomers are in the brain (Tomic et al., 2009; Pop et al., 2010a). Similar to fibrillar Aβ, oligomeric Aβ can be measured in CSF, where levels are inversely related to levels in the brain (Head et al., 2010).

Aβ can also aggregate in the cerebral blood vessel walls and cause *cerebrovascular pathology* (Prior et al., 1996; Attems, 2005; Herzig et al., 2006). This type of deposition is referred to as cerebral amyloid angiopathy (CAA; **Figures 1C,D**). Typically CAA is composed of the shorter Aβ 1-40 peptide (Wisniewski et al., 1996; Attems, 2005; Herzig et al., 2006). Both humans and canines exhibit CAA pathology, with a particular vulnerability in the occipital cortex (Attems et al., 2005). CAA impairs the blood brain barrier, vascular function, and can cause microhemorrhages (Uchida et al., 1990; Prior et al., 1996; Deane and Zlokovic, 2007). Because of these complications, CAA may contribute to cognitive decline in both humans (Ellis et al., 1996; Rensink et al., 2003; Nicoll et al., 2004; Attems, 2005) and canines (Giaccone et al., 1990; Uchida et al., 1990, 1991; Head, 2013). Much like humans, canines experience microhemorrhages with age (Uchida et al., 1991). These cerebral hemorrhages are present in both animals with and without CAA, but are more common in those with the blood vessel pathology (Uchida et al., 1991). Given the significant overlap of cerebrovascular pathology with AD, the spontaneous accumulation of CAA in dogs also offers as yet, an underappreciated model system to test the effects of cerebrovascular pathology on cognition and AD neuropathology.

Aβ deposition may lead to oxidative damage or *vice versa*, oxidative damage may lead to Aβ (Butterfield, 1997). Ultimately, *oxidative damage* accumulates with age and can lead to neuronal dysfunction and thus impact cognition (Butterfield et al., 2001). Oxidative damage occurs over time due to the overproduction of reactive oxygen species (ROS) produced primarily by mitochondria. When there is an overabundance of ROS, various mechanisms including production and release of endogenous antioxidants are in place to restore a homeostatic balance. However, ROS overproduction may exceed the levels or production rate of endogenous antioxidants and result in oxidative damage to proteins, lipids, and nucleotides. Oxidative damage can be measured by the amount of protein oxidation (carbonyl

groups), 4-hydroxynonenal, lipofuscin, lipofuscin-like pigments, and malondialdehyde (lipid peroxidation). Further, 8-hydroxy-2′-deoxyguanosine (8OHdG) can be measured to detect DNA/RNA oxidation.

While oxidative damage occurs with normal aging, it is more pronounced in AD (Smith et al., 1991, 1996, 2000; Ames et al., 1993; Lovell et al., 1999; Montine et al., 2002; Pratico et al., 2002; Butterfield et al., 2007; Lovell and Markesbery, 2008), and similar patterns are seen in canines. In the canine model, there is evidence that ROS production is higher than in younger animals. In mitochondria isolated from aged canine brain, there is an increased production of ROS compared to mitochondria isolated from young animals (Head et al., 2009). Canines also experience an accumulation of carbonyl groups with age (Head et al., 2002; Skoumalova et al., 2003). Lipid peroxidation is exhibited in old dogs, measured by 4-hydroxynonenal (Papaioannou et al., 2001; Rofina et al., 2004, 2006; Hwang et al., 2008a), lipofuscin (Rofina et al., 2006), lipofuscin-like pigments (Papaioannou et al., 2001; Rofina et al., 2004), or malondialdehyde (Head et al., 2002). Increased 8OHdG in aged canines has also been reported (Rofina et al., 2006; Cotman and Head, 2008). In particular, increased protein oxidation and lipid peroxidation (lipofuscin-like pigment) correlates with cognitive decline in dogs (Skoumalova et al., 2003; Rofina et al., 2004, 2006). Given that canines exhibit age-associated oxidative damage in the brain that correlates with poorer cognition, these animals are suitable to study antioxidant treatment/prevention strategies.

One hallmark AD pathology canines do not produce is NFTs (Selkoe et al., 1987; Russell et al., 1992). While no research to date has observed NFTs in the canine brain, the increased phosphorylation seen at some sites of tau in AD cases also occurs in cognitively impaired canines (Kuroki et al., 1997; Wegiel et al., 1998; Papaioannou et al., 2001; Head et al., 2005; Pugliese et al., 2006b). This lack of NFT pathology could possibly be due to significant differences in the tau protein sequence between canines and humans[3]. However, an advantage to dogs not accumulating NFTs is that they serve as a model that is selective for Aβ pathology and ideally suited for testing interventions that target this toxic protein.

TREATMENT STUDIES IN AGED DOGS AND PREDICTING HUMAN CLINICAL TRIALS

Several studies have tested therapeutic strategies using the canine model of aging and AD with both cognitive and neuropathological outcome measures (**Table 2**). Several of these involve dietary and/or environmental manipulations. One of the earliest studies to develop a treatment for cognitive dysfunction in aged dogs tested *an antioxidant-rich diet in combination with behavioral enrichment.* The behavioral enrichment included increased exercise, interaction with other dogs, and cognitive enrichment (Cotman et al., 2002; Milgram et al., 2002a, 2004, 2005). The diet included vitamins E and C, fruits and vegetables, lipoic acid and carnitine. Compared to control animals, those receiving an antioxidant-rich diet committed fewer errors during landmark acquisition and retention tasks (Milgram et al., 2002a) as well as oddity discrimination tasks (Cotman et al., 2002). Treatment with an antioxidant diet and behavioral enrichment resulted in improved performance during black and white object discrimination and reversal (Milgram et al., 2005). Pop et al. (2010b) found dogs provided with both behavioral enrichment and an antioxidant diet have an overall reduction in Aβ pathology across multiple regions of the brain. However, when looking at group treatment effects, only the antioxidant-treated animals had a significant reduction in Aβ plaque pathology. Additionally, the combination treatment approach of behavioral enrichment and an antioxidant-rich diet in aged canines was unable to reduce existing brain Aβ (Pop et al., 2010b). While plaque load was affected by the dual intervention, soluble and insoluble Aβ 1-40 was not affected, and only soluble levels of Aβ 1-42 were lowered specifically in the prefrontal cortex. A trend toward a significant decrease in oligomers specifically in the parietal cortex was observed in canines receiving the combined treatment (Pop et al., 2010b). Interestingly, the combination group also showed reduced oxidative damage (Opii et al., 2008) with the antioxidant diet group alone showing reduced mitochondrial dysfunction (Head et al., 2009). Further, the behavioral enrichment group, independent of the antioxidant diet treatment showed less neuron loss in the hippocampus (Siwak-Tapp et al., 2008) as well as improved levels of brain derived growth factor (Fahnestock et al., 2010).

Supplemental medium-chain TAG (MCT) increases ketone levels in the brain, and these ketones can in turn be used as an alternative energy source. Pan et al. (2010) measured cognitive effects seen due to this supplement on the landmark discrimination, oddity discrimination, and two choice egocentric spatial learning tasks. Results indicated aged dogs given a diet with MCT supplementation performed better than those receiving a control diet in all tasks (Pan et al., 2010).

In contrast, fewer benefits on cognition were observed in a study using a *medical food cocktail* (Head et al., 2012). Dogs receiving a combination cocktail containing an extract of turmeric containing 95% curcuminoids, an extract of green tea containing 50% epigallocatechin gallate, N-acetyl cysteine, R-alpha lipoic acid and an extract of black pepper containing 95% piperine exhibited fewer errors compared to control animals during the landmark task indicating improved spatial attention. However, other areas of cognition were unaffected and brain Aβ remained unchanged (Head et al., 2012).

In 2008, a therapeutic approach that directly targeted Aβ reduction was explored in which aged beagles were actively immunized with fibrillar Aβ1-42 for 2 years (Immunized - IMM) based upon previous work in transgenic mouse models of AD (Schenk et al., 1999). This *immunotherapy* approach led to no improvement in cognitive function, but interestingly a long term maintenance of executive function was noted based on error scores from the size reversal learning task (Head et al., 2008). However, significant benefits to brain pathology were observed in the IMM dogs who showed significantly decreased Aβ plaque load in prefrontal, entorhinal, and occipital cortical regions, as well as reduced CAA (Head et al., 2008). While soluble and insoluble brain Aβ 1-40 and 42 significantly decreased in treated canines, there was no significant reduction in soluble oligomers. This study suggests that reducing

[3]http://www.ensembl.org/Canis_familiaris/

Table 2 | Cognitive outcomes of treatment studies in aging dogs*.

Treatment	Sample size and age	Landmark discrimination	Oddity discrimination	Size discrimination	Size reversal	Black white discrimination	Black/white reversal	Spatial memory	Questionnaire	Publication
Antioxidant diet	28 old	Improved	Improved	Improved	Improved	Improved	Improved	Improved	Not assessed	Cotman et al. (2002),
Behavioral enrichment	(8–13 years)	Not assessed	Not assessed	Improved	Improved	Improved	Improved	Improved	Not assessed	Milgram et al. (2004, 2005)
Antioxidant diet + behavioral enrichment		Improved	Improved	Improved	Improved	Improved	Improved	Improved	Not assessed	
MCT dietary supplement	24 old (9–10 years)	Not improved	Impaired	Not assessed	Not assessed	Not assessed	Not assessed	Impaired	Not assessed	Pan et al. (2010)
Medical food cocktail	18 old (8–9 years)	Improved	Not improved	Not improved	Not improved	Not improved	Not improved	Not improved	Not assessed	Head et al. (2012)
Atorvastatin	10 old (9–13 years)	Not assessed	Not assessed	Not improved	Impaired	Not improved	Not improved	Not improved	Not assessed	Murphy et al. (2010)
Fibrillar Aβ1-42 immunotherapy	20 old (8–13 years)	Not improved	Not improved	Not improved	Maintained	Not improved	Maintained	Not improved	Not assessed	Head et al. (2008)
Fibrillar Aβ 1-40 and x-40 immunotherapy	12 old (11–18 years)	Not assessed	Not assessed	Not assessed	Not assessed	Not assessed	Not assessed	Not assessed	Improved	Bosch et al. (2013)

Not an exhaustive list.

or eliminating pre-existing Aβ in aging dogs is not sufficient to improve cognition.

Outcomes from the longitudinal dog vaccination study are similar to reports of the clinical trial in patients with AD where no differences between antibody responders and placebo groups on several cognitive and disability scales was observed. A small number of patients enrolled in the AN1792 study have come to autopsy and show Aβ plaque reduction without any effect on the extent of NFT or CAA (Nicoll et al., 2003; Ferrer et al., 2004; Masliah et al., 2005). Further, the frontal cortex showed the largest response to immunotherapy (Masliah et al., 2005), which is similar to our observations in the dog. The most recent autopsy study of eight patients that were in the AN1792 study further confirm reduced Aβ pathology in response to treatment, 5 years after the last injection (Holmes et al., 2008). However, reduction of brain Aβ did not slow disease progression and seven of eight patients had severe end stage dementia prior to death. (Gilman et al., 2005). Interestingly, a composite score of a neuropsychological test battery indicated "less worsening" of decline in antibody responders after 12 months and an improvement in the memory domain (Gilman et al., 2005).

Bosch et al. (2013) recently showed benefits of an active fibrillar $A\beta_{40}$ and $A\beta_{x-40}$ combination vaccine on cognition in aged housed beagles and pet dogs treated for 51 days. Over the course of treatment, cognitive evaluations by questionnaire were given at 31 days post treatment and at the end of treatment. Immunized animals showed a significant improvement in cognitive evaluation scores at both 31 and 51 days post treatment compared to pre-immunized scores (Bosch et al., 2013). Differences in the formulation, the outcome measures or the source of animals may explain the positive effects in the Bosch study compared with the previous beagle vaccine studies.

Several studies in the aged dog have tested the effects of drugs already approved for use in humans, with novel applications to brain aging. For example, several cross-sectional or case-control epidemiological studies revealed a striking link between *cholesterol-lowering drugs* (e.g., statins and others) and a 20–70% reduction in risk of developing AD (Jick et al., 2000; Wolozin et al., 2000, 2007; Hajjar et al., 2002; Rockwood et al., 2002; Rodriguez et al., 2002; Zamrini et al., 2004; Dufouil et al., 2005). Modest cognitive benefits have been reported in preliminary AD clinical trials with simvastatin (Simons et al., 2002) and atorvastatin (Sparks et al., 2005a,b, 2006a,b). In particular, AD patients with mild to moderate dementia who were treated with 80 mg/day atorvastatin had significantly improved scores on one measure of cognition Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-Cog) at 6 months of treatment, with smaller non-significant benefits at 12 months (Sparks et al., 2005b).

Statins may reduce the risk of incident AD through the prevention of Aβ production (Simons et al., 1998; Hartmann, 2001). In rodent models, treatment with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) or statins reduces Aβ (Petanceska et al., 2002). However, rodents respond to statin treatment by massively upregulating HMG-CoA reductase levels (Fears et al., 1980; Alberts, 1990; Todd and Goa, 1990; Thelen et al., 2006). To compensate, long-term studies in rodent often employ physiologically excessive doses, making it difficult to translate the results of these studies into human trials.

The dog model is particularly useful to study chronic statin treatment, given similarities with humans in terms of dose requirements, responsiveness, drug handling, and metabolism (Gerson et al., 1989; Alberts, 1990). For example, 12 dogs were treated with 80 mg/day of atorvastatin for 14.5 months (Murphy et al., 2010). Peripheral levels of cholesterol, low density lipoproteins, triglycerides and high density lipoproteins were reduced in treated dogs. Surprisingly, a transient impairment in reversal learning was observed, suggesting prefrontal dysfunction. Spatial memory remained unchanged up to over a year of treatment. The lack of cognitive benefits of treatment was also reflected by a lack of reduction in plasma, CSF, and brain Aβ. Interestingly, BACE1 protein level was decreased in the brains of atorvastatin-treated dogs. This intriguing outcome may suggest that statins might be more useful to prevent the production of Aβ through lowering BACE1 if started in animals in middle age, consistent with human studies indicating that middle-aged individuals using statins are protected from AD.

More recent work on the brain from statin-treated aged dogs suggests that additional benefits of atorvastatin include reducing oxidative damage and upregulating endogenous protective pathways. Thus, statins may have multiple benefits to the brain by affecting several pathways impaired by aging (Barone et al., 2011, 2012; Martin et al., 2011a; Butterfield et al., 2012). Aged dogs are a unique model that may provide novel insights and translational data to predict outcomes of statin use in human clinical trials.

SUMMARY

Aged dogs capture many features of human aging and AD including cognitive decline and neuropathology. Canine studies show that multi-targeted approaches may be more beneficial than single pathway manipulations (e.g., antioxidants combined with behavioral enrichment vs. Aβ vaccine). Further, prevention studies could be accomplished in a 5-year period to test the effects of an intervention on the development of cognitive decline and neuropathology. Interestingly, an immunotherapy study in aged dogs illustrates the predictive validity of using this model system as aged dogs did not show cognitive improvements with an Aβ vaccine despite showing significant brain Aβ reductions, much like reports in the AD clinical trial. The canine model has numerous advantages as described above, however, systematic cognitive testing can be a lengthy and costly (given per diem rates) process and requires significant technical support. Still, the canine model should be considered an option since it is less involved and costly than a human clinical prevention study. Overall, using the dog as a preclinical model for testing preventive approaches for AD may be a useful step that complements work in rodents and non-human primates.

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REFERENCES

Adams, B., Chan, A., Callahan, H., and Milgram, N. W. (2000). The canine as a model of human cognitive aging: recent developments. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 24, 675–692. doi: 10.1016/S0278-5846(00)00101-9

Aisen, P. S., Cummings, J., and Schneider, L. S. (2012). Symptomatic and nonamyloid/tau based pharmacologic treatment for Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2, a006395. doi: 10.1101/cshperspect.a006395

Alavi, A., Newberg, A. B., Souder, E., and Berlin, J. A. (1993). Quantitative analysis of PET and MRI data in normal aging and Alzheimer's disease: atrophy weighted total brain metabolism and absolute whole brain metabolism as reliable discriminators. *J. Nucl. Med.* 34, 1681–1687.

Alberts, A. W. (1990). Lovastatin and simvastatin – inhibitors of HMG CoA reductase and cholesterol biosynthesis. *Cardiology* 77, 14–21. doi: 10.1159/000174688

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7915–7922. doi: 10.1073/pnas.90.17.7915

Arnsten, A. F. T., and Goldman-Rakic, P. S. (1985). Alpha 2-adrenergic mechanisms in prefrontal cortex associated with cognitive decline in aged nonhuman primates. *Science* 230, 1273–1276. doi: 10.1126/science.2999977

Attems, J. (2005). Sporadic cerebral amyloid angiopathy: pathology, clinical implications, and possible pathomechanisms. *Acta Neuropathol.* 110, 345–359. doi: 10.1007/s00401-005-1074-9

Attems, J., Jellinger, K. A., and Lintner, F. (2005). Alzheimer's disease pathology influences severity and topographical distribution of cerebral amyloid angiopathy. *Acta Neuropathol.* 110, 222–231. doi: 10.1007/s00401-005-1064-y

Axelsson, E., Ratnakumar, A., Arendt, M. L., Maqbool, K., Webster, M. T., Perloski, M., et al. (2013). The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature* 495, 360–364. doi: 10.1038/nature11837

Azizeh, B. Y., Head, E., Ibrahim, M. A., Torp, R., Tenner, A. J., Kim, R. C., et al. (2000). Molecular dating of senile plaques in the brains of individuals with down syndrome and in aged dogs. *Exp. Neurol.* 163, 111–122. doi: 10.1006/exnr.2000.7359

Ballard, C. G., Greig, N. H., Guillozet-Bongaarts, A. L., Enz, A., and Darvesh, S. (2005). Cholinesterases: roles in the brain during health and disease. *Curr. Alzheimer Res.* 2, 307–318. doi: 10.2174/1567205054367838

Barone, E., Cenini, G., Di Domenico, F., Martin, S., Sultana, R., Mancuso, C., et al. (2011). Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: a novel mechanism of action. *Pharmacol. Res.* 63, 172–180. doi: 10.1016/j.phrs.2010.12.007

Barone, E., Mancuso, C., Di Domenico, F., Sultana, R., Murphy, M. P., Head, E., et al. (2012). Biliverdin reductase-A: a novel drug target for atorvastatin in a dog pre-clinical model of Alzheimer disease. *J. Neurochem.* 120, 135–146. doi: 10.1111/j.1471-4159.2011.07538.x

Bernedo, V., Insua, D., Suarez, M. L., Santamarina, G., Sarasa, M., and Pesini, P. (2009). β-amyloid cortical deposits are accompanied by the loss of serotonergic neurons in the dog. *J. Comp. Neurol.* 513, 417–429. doi: 10.1002/cne.21985

Blizard, D. A., Klein, L. C., Cohen, R., and McClearn, G. E. (2003). A novel mouse-friendly cognitive task suitable for use in aging studies. *Behav. Genet.* 33, 181–189. doi: 10.1023/A:1022510119598

Bobinski, M., Wegiel, J., Tarnawski, M., Bobinski, M., Reisberg, B., de Leon, M. J., et al. (1997). Relationships between regional neuronal loss and neurofibrillary changes in the hippocampal formation and duration and severity of Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 56, 414–420. doi: 10.1097/00005072-199704000-00010

Bosch, M. N., Gimeno-Bayon, J., Rodriguez, M. J., Pugliese, M., and Mahy, N. (2013). Rapid improvement of canine cognitive dysfunction with immunotherapy designed for Alzheimer's disease. *Curr. Alzheimer Res.* 10, 482–493. doi: 10.2174/15672050113109990129

Bosch, M. N., Pugliese, M., Gimeno-Bayon, J., Rodriguez, M. J., and Mahy, N. (2012). Dogs with cognitive dysfunction syndrome: a natural model of Alzheimer's disease. *Curr. Alzheimer Res.* 9, 298–314. doi: 10.2174/156720512800107546

Boutet, I., Milgram, N. W., and Freedman, M. (2007). Cognitive decline and human (*Homo sapiens*) aging: an investigation using a comparative neuropsychological approach. *J. Comp. Psychol.* 121, 270–281. doi: 10.1037/0735-7036.121.3.270

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82, 239–259. doi: 10.1007/BF00308809

Butterfield, D. A. (1997). β-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. *Chem. Res. Toxicol.* 10, 495–506. doi: 10.1021/tx960130e

Butterfield, D. A., Barone, E., Di Domenico, F., Cenini, G., Sultana, R., Murphy, M. P., et al. (2012). Atorvastatin treatment in a dog preclinical model of Alzheimer's disease leads to up-regulation of haem oxygenase-1 and is associated with reduced oxidative stress in brain. *Int. J. Neuropsychopharmacol.* 15, 981–987. doi: 10.1017/S1461145711001118

Butterfield, D. A., Drake, J., Pocernich, C., and Castegna, A. (2001). Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β-peptide. *Trends Mol. Med.* 7, 548–554. doi: 10.1016/S1471-4914(01)02173-6

Butterfield, D. A., Reed, T., Newman, S. F., and Sultana, R. (2007). Roles of amyloid β-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. *Free Radic. Biol. Med.* 43, 658–677. doi: 10.1016/j.freeradbiomed.2007.05.037

Chan, A. D., Nippak, P. M., Murphey, H., Ikeda-Douglas, C. J., Muggenburg, B., Head, E., et al. (2002). Visuospatial impairments in aged canines (*Canis familiaris*): the role of cognitive-behavioral flexibility. *Behav. Neurosci.* 116, 443–454. doi: 10.1037/0735-7044.116.3.443

Christie, L. A., Studzinski, C. M., Araujo, J. A., Leung, C. S., Ikeda-Douglas, C. J., Head, E., et al. (2005). A comparison of egocentric and allocentric age-dependent spatial learning in the beagle dog. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 361–369. doi: 10.1016/j.pnpbp.2004.12.002

Colle, M.-A., Hauw, J.-J., Crespeau, F., Uchiara, T., Akiyama, H., Checler, F., et al. (2000). Vascular and parenchymal Ab deposition in the aging dog: correlation with behavior. *Neurobiol. Aging* 21, 695–704. doi: 10.1016/S0197-4580(00)00113-5

Cotman, C. W., and Head, E. (2008). The canine (dog) model of human aging and disease: dietary, environmental and immunotherapy approaches. *J. Alzheimers Dis.* 15, 685–707.

Cotman, C. W., Head, E., Muggenburg, B. A., Zicker, S., and Milgram, N. W. (2002). Brain aging in the canine: a diet enriched in antioxidants reduces cognitive dysfunction. *Neurobiol. Aging* 23, 809–818. doi: 10.1016/S0197-4580(02)00073-8

Cronin-Golomb, A. (2001). "Color vision, object recognition, and spatial localization in aging and Alzheimer's disease," in *Functional Neurobiology of Aging*, eds P. R. Hof and C. V. Mobbs (San Diego: Academic Press), 517–529.

Cummings, B. J., Head, E., Afagh, A. J., Milgram, N. W., and Cotman, C. W. (1996a). β-amyloid accumulation correlates with cognitive dysfunction in the aged canine. *Neurobiol. Learn. Mem.* 66, 11–23. doi: 10.1006/nlme.1996.0039

Cummings, B. J., Head, E., Ruehl, W., Milgram, N. W., and Cotman, C. W. (1996b). The canine as an animal model of human aging and dementia. *Neurobiol. Aging* 17, 259–268. doi: 10.1016/0197-4580(95)02060-8

Cummings, B. J., Su, J. H., Cotman, C. W., White, R., and Russell, M. J. (1993). β-amyloid accumulation in aged canine brain: a model of plaque formation in Alzheimer's disease. *Neurobiol. Aging* 14, 547–560. doi: 10.1016/0197-4580(93)90038-D

Deane, R., and Zlokovic, B. V. (2007). Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease. *Curr. Alzheimer Res.* 4, 191–197. doi: 10.2174/156720507780362245

de Veer, M. W., Gallup, G. G. Jr., Theall, L. A., van den Bos, R., and Povinelli, D. J. (2003). An 8-year longitudinal study of mirror self-recognition in chimpanzees (Pan troglodytes). *Neuropsychologia* 41, 229–234. doi: 10.1016/S0028-3932(02)00153-7

Du, A. T., Schuff, N., Chao, L. L., Kornak, J., Ezekiel, F., Jagust, W. J., et al. (2005). White matter lesions are associated with cortical atrophy more than entorhinal and hippocampal atrophy. *Neurobiol. Aging* 26, 553–559. doi: 10.1016/j.neurobiolaging.2004.05.002

Dufouil, C., Richard, F., Fievet, N., Dartigues, J. F., Ritchie, K., Tzourio, C., et al. (2005). APOE genotype, cholesterol level, lipid-lowering treatment, and dementia: the Three-City Study. *Neurology* 64, 1531–1538. doi: 10.1212/01.WNL.0000160114.42643.31

Ellis, R. J., Olichney, J. M., Thal, L. J., Mirra, S. S., Morris, J. C., Beekly, D., et al. (1996). Cerebral amyloid angiopathy in the brains of patients with Alzheimer's disease: the CERAD experience, Part, X. V. *Neurology* 46, 1592–1596. doi: 10.1212/WNL.46.6.1592

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., et al. (1998). Neurogenesis in the adult human hippocampus. *Nat. Med.* 4, 1313–1317. doi: 10.1038/3305

Ezekiel, F., Chao, L., Kornak, J., Du, A. T., Cardenas, V., Truran, D., et al. (2004). Comparisons between global and focal brain atrophy rates in normal aging and Alzheimer disease: boundary shift integral versus tracing of the entorhinal cortex and hippocampus. *Alzheimer Dis. Assoc. Disord.* 18, 196–201.

Fahnestock, M., Marchese, M., Head, E., Pop, V., Michalski, B., Milgram, W. N., et al. (2010). BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. *Neurobiol. Aging* 33, 546–554. doi: 10.1016/j.neurobiolaging.2010.03.019

Fears, R., Richards, D. H., and Ferres, H. (1980). The effect of compactin, a potent inhibitor of 3-hydroxy-3-methylglutaryl co-enzyme-A reductase activity, on cholesterogenesis and serum cholesterol levels in rats and chicks. *Atherosclerosis* 35, 439–449. doi: 10.1016/0021-9150(80)90185-9

Ferrer, I., Boada Rovira, M., Sanchez Guerra, M. L., Rey, M. J., and Costa-Jussa, F. (2004). Neuropathology and pathogenesis of encephalitis following amyloid-β immunization in Alzheimer's disease. *Brain Pathol.* 14, 11–20. doi: 10.1111/j.1750-3639.2004.tb00493.x

Fox, M. W., and Weisman, R. (1970). Development of responsiveness to a social releaser in the dog: effects of age and hunger. *Dev. Psychobiol.* 2, 277–280. doi: 10.1002/dev.420020414

Freedman, M., and Oscar-Berman, M. (1989). Spatial and visual learning deficits in Alzheimer's disease and Parkinson's disease. *Brain Cogn.* 11, 114–126. doi: 10.1016/0278-2626(89)90009-2

Gallagher, M., and Rapp, P. R. (1997). The use of animal models to study the effects of aging on cognition. *Annu. Rev. Psychol.* 48, 339–370. doi: 10.1146/annurev.psych.48.1.339

Gallup, G. G. Jr. (1968). Mirror-image stimulation. *Psychological. Bull.* 70, 782–793. doi: 10.1037/h0026777

Gerson, R. J., MacDonald, J. S., Alberts, A. W., Kornbrust, D. J., Majka, J. A., Stubbs, R. J., et al. (1989). Animal safety and toxicology of simvastatin and related hydroxy-methylglutaryl-coenzyme a reductase inhibitors. *Am. J. Med.* 87, 28S–38S. doi: 10.1016/S0002-9343(89)80596-0

Giaccone, G., Verga, L., Finazzi, M., Pollo, B., Tagliavini, F., Frangione, B., et al. (1990). Cerebral preamyloid deposits and congophilic angiopathy in aged dogs. *Neurosci. Lett.* 114, 178–183. doi: 10.1016/0304-3940(90)90068-K

Gilman, S., Koller, M., Black, R. S., Jenkins, L., Griffith, S. G., Fox, N. C., et al. (2005). Clinical effects of Aβ immunization (AN1792) in patients with AD in an interrupted trial. *Neurology* 64, 1553–1562. doi: 10.1212/01.WNL.0000159740.16984.3C

Gonzalez-Soriano, J., Marin Garcia, P., Contreras-Rodriguez, J., Martinez-Sainz, P., and Rodriguez-Veiga, E. (2001). Age-related changes in the ventricular system of the dog brain. *Ann. Anat.* 183, 283–291. doi: 10.1016/S0940-9602(01)80236-3

Hajjar, I., Schumpert, J., Hirth, V., Wieland, D., and Eleazer, G. P. (2002). The impact of the use of statins on the prevalence of dementia and the progression of cognitive impairment. *J. Gerontol. A Biol. Sci. Med. Sci.* 57, M414–M418. doi: 10.1093/gerona/57.7.M414

Hardy, J. (2006). Alzheimer's disease: the amyloid cascade hypothesis: an update and reappraisal. *J. Alzheimers Dis.* 9(Suppl. 3), 151–153.

Hardy, J. A., and Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256, 184–185. doi: 10.1126/science.1566067

Hartmann, T. (2001). Cholesterol, A β and Alzheimer's disease. *Trends Neurosci.* 24 (Suppl. 11), S45–S48. doi: 10.1016/S0166-2236(01)00008-X

Head, E. (2001). Brain aging in dogs: parallels with human brain aging and alzheimer's disease. *Vet. Ther.* 2, 247–260.

Head, E. (2013). A canine model of human aging and Alzheimer's disease. *Biochim. Biophys. Acta* 1832, 1384–1389. doi: 10.1016/j.bbadis.2013.03.016

Head, E., Callahan, H., Cummings, B. J., Cotman, C. W., Ruehl, W. W., Muggenberg, B. A., et al. (1997). Open field activity and human interaction as a function of age and breed in dogs. *Physiol. Behav.* 62, 963–971. doi: 10.1016/S0031-9384(97)00198-4

Head, E., Callahan, H., Muggenburg, B. A., Cotman, C. W., and Milgram, N. W. (1998a). Visual-discrimination learning ability and β-amyloid accumulation in the dog. *Neurobiol. Aging* 19, 415–425. doi: 10.1016/S0197-4580(98)00084-0

Head, E., McCleary, R., Hahn, F., Milgram, N. W., and Cotman, C. W. (1998b). "Predicting the presence and location of amyloid deposition in a canine model of human aging and dementia using logistic regression analyses," *Proceedings of the sixth International Conference on Alzheimer's Disease and Related Disorders*, Amsterdam, Holland.

Head, E., Liu, J., Hagen, T. M., Muggenburg, B. A., Milgram, N. W., Ames, B. N., et al. (2002). Oxidative damage increases with age in a canine model of human brain aging. *J. Neurochem.* 82, 375–381. doi: 10.1046/j.1471-4159.2002.00969.x

Head, E., McCleary, R., Hahn, F. F., Milgram, N. W., and Cotman, C. W. (2000). Region-specific age at onset of β-amyloid in dogs. *Neurobiol. Aging* 21, 89–96. doi: 10.1016/S0197-4580(00)00093-2

Head, E., Mehta, R., Hartley, J., Kameka, M., Cummings, B. J., Cotman, C. W., et al. (1995). Spatial learning and memory as a function of age in the dog. *Behav. Neurosci.* 109, 851–858. doi: 10.1037/0735-7044.109.5.851

Head, E., and Milgram, N. W. (1992). Changes in spontaneous behavior in the dog following oral administration of L-deprenyl. *Pharmacol. Biochem. Behav.* 43, 749–757. doi: 10.1016/0091-3057(92)90404-4

Head, E., Milgram, N. W., and Cotman, C. W. (2001). "Neurobiological models of aging in the dog and other vertebrate species," in *Functional Neurobiology of Aging*, eds P. Hof and C. Mobbs (San Diego: Academic Press), 457–468.

Head, E., Moffat, K., Das, P., Sarsoza, F., Poon, W. W., Landsberg, G., et al. (2005). β-Amyloid deposition and tau phosphorylation in clinically characterized aged cats. *Neurobiol. Aging* 26, 749–763. doi: 10.1016/j.neurobiolaging.2004.06.015

Head, E., Murphey, H. L., Dowling, A. L., McCarty, K. L., Bethel, S. R., Nitz, J. A., et al. (2012). A combination cocktail improves spatial attention in a canine model of human aging and Alzheimer's disease. *J. Alzheimers Dis.* 32, 1029–1042. doi: 10.3233/JAD-2012-120937

Head, E., Nukala, V. N., Fenoglio, K. A., Muggenburg, B. A., Cotman, C. W., and Sullivan, P. G. (2009). Effects of age, dietary, and behavioral enrichment on brain mitochondria in a canine model of human aging. *Exp. Neurol.* 220, 171–176. doi: 10.1016/j.expneurol.2009.08.014

Head, E., Pop, V., Sarsoza, F., Kayed, R., Beckett, T. L., Studzinski, C. M., et al. (2010). Amyloid-β peptide and oligomers in the brain and cerebrospinal fluid of aged canines. *J. Alzheimers Dis.* 20, 637–646. doi: 10.3233/JAD-2010-1397

Head, E., Pop, V., Vasilevko, V., Hill, M., Saing, T., Sarsoza, F., et al. (2008). A two-year study with fibrillar β-amyloid (Aβ) immunization in aged canines: effects on cognitive function and brain Aβ. *J. Neurosci.* 28, 3555–3566. doi: 10.1523/JNEUROSCI.0208-08.2008

Herzig, M. C., Van Nostrand, W. E., and Jucker, M. (2006). Mechanism of cerebral β-amyloid angiopathy: murine and cellular models. *Brain Pathol.* 16, 40–54. doi: 10.1111/j.1750-3639.2006.tb00560.x

Holmes, C., Boche, D., Wilkinson, D., Yadegarfar, G., Hopkins, V., Bayer, A., et al. (2008). Long-term effects of Aβ42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. *Lancet* 372, 216–223. doi: 10.1016/S0140-6736(08)61075-2

Hwang, I. K., Yoon, Y. S., Yoo, K. Y., Li, H., Choi, J. H., Kim, D. W., et al. (2008a). Differences in lipid peroxidation and Cu,Zn-Superoxide dismutase in the hippocampal CA1 region between adult and aged dogs. *J. Vet. Med. Sci.* 70, 273–277. doi: 10.1292/jvms.70.273

Hwang, I. K., Yoon, Y. S., Yoo, K. Y., Li, H., Sun, Y., Choi, J. H., et al. (2008b). Sustained expression of parvalbumin immunoreactivity in the hippocampal CA1 region and dentate gyrus during aging in dogs. *Neurosci. Lett.* 434, 99–103. doi: 10.1016/j.neulet.2008.01.035

Insua, D., Suarez, M. L., Santamarina, G., Sarasa, M., and Pesini, P. (2010). Dogs with canine counterpart of Alzheimer's disease lose noradrenergic neurons. *Neurobiol. Aging* 31, 625–635. doi: 10.1016/j.neurobiolaging.2008.05.014

Jick, H., Zornberg, G. L., Jick, S. S., Seshadri, S., and Drachman, D. A. (2000). Statins and the risk of dementia. *Lancet* 356, 1627–1631. doi: 10.1016/S0140-6736(00)03155-X

Johnstone, E. M., Chaney, M. O., Norris, F. H., Pascual, R., and Little, S. P. (1991). Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. *Brain Res. Mol. Brain Res.* 10, 299–305. doi: 10.1016/0169-328X(91)90088-F

Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., et al. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300, 486–489. doi: 10.1126/science.1079469

Kimotsuki, T., Nagaoka, T., Yasuda, M., Tamahara, S., Matsuki, N., and Ono, K. (2005). Changes of magnetic resonance imaging on the brain in beagle dogs with aging. *J. Vet. Med. Sci.* 67, 961–967. doi: 10.1292/jvms.67.961

Knobloch, M., and Mansuy, I. M. (2008). Dendritic spine loss and synaptic alterations in Alzheimer's disease. *Mol. Neurobiol.* 37, 73–82. doi: 10.1007/s12035-008-8018-z

Kowalska, D. M. (1995). Effects of hippocampal lesions on spatial delayed responses in dog. *Hippocampus* 5, 363–370. doi: 10.1002/hipo.450050409

Kramer, J. H., and Quitania, L. (2007). "Bedside frontal lobe testing," in *The Human Frontal Lobes,* 3rd Edn, eds B. L. Miller and J. L. Cummings (New York: The Guilford Press), 279–291.

Kuroki, K., Uchida, K., Kiatipattanasakul, W., Nakamura, S., Yamaguchi, R., Nakayama, H., et al. (1997). Immunohistochemical detection of tau proteins in various non-human animal brains. *Neuropathology* 17, 174–180. doi: 10.1111/j.1440-1789.1997.tb00034.x

Lai, Z. C., Moss, M. B., Killiany, R. J., Rosene, D. L., and Herndon, J. G. (1995). Executive system dysfunction in the aged monkey: spatial and object reversal learning. *Neurobiol. Aging* 16, 947–954. doi: 10.1016/0197-4580(95)02014-4

Landsberg, G., and Araujo, J. A. (2005). Behavior problems in geriatric pets. *Vet. Clin. North Am. Small Anim. Pract.* 35, 675–698. doi: 10.1016/j.cvsm.2004.12.008

Landsberg, G., and Ruehl, W. (1997). Geriatric behavioral problems. *Vet. Clin. North Am. Small Anim. Pract.* 27, 1537–1559.

Landsberg, G. M., Nichol, J., and Araujo, J. A. (2012). Cognitive dysfunction syndrome: a disease of canine and feline brain aging. *Vet. Clin. North Am. Small Anim. Pract.* 42, 749–768. doi: 10.1016/j.cvsm.2012.04.003

Lezak, M. D., Howieson, D. B., and Loring, D. W. (2004). *Neuropsychological Assessment.* 4th Edn. New York: Oxford University Press.

Lovell, M. A., Gabbita, S. P., and Markesbery, W. R. (1999). Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. *J. Neurochem.* 72, 771–776. doi: 10.1046/j.1471-4159.1999.0720771.x

Lovell, M. A., and Markesbery, W. R. (2008). Oxidatively modified RNA in mild cognitive impairment. *Neurobiol. Dis.* 29, 169–175. doi: 10.1016/j.nbd.2007.07.030

Markesbery, W. R., Schmitt, F. A., Kryscio, R. J., Davis, D. G., Smith, C. D., and Wekstein, D. R. (2006). Neuropathologic substrate of mild cognitive impairment. *Arch. Neurol.* 63, 38–46. doi: 10.1001/archneur.63.1.38

Martin, S. B., Cenini, G., Barone, E., Dowling, A. L., Mancuso, C., Butterfield, D. A., et al. (2011a). Coenzyme Q10 and cognition in atorvastatin treated dogs. *Neurosci. Lett.* 501, 92–95. doi: 10.1016/j.neulet.2011.06.054

Martin, S. B., Dowling, A. L., and Head, E. (2011b). Therapeutic interventions targeting β amyloid pathogenesis in an aging dog model. *Curr. Neuropharmacol.* 9, 651–661. doi: 10.2174/157015911798376217

Masliah, E., Hansen, L., Adame, A., Crews, L., Bard, F., Lee, C., et al. (2005). Aβ vaccination effects on plaque pathology in the absence of encephalitis in Alzheimer disease. *Neurology* 64, 129–131. doi: 10.1212/01.WNL.0000148590.39911.DF

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E. M. (1984). Clinical Diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services task force on Alzheimer's disease. *Neurology* 34, 939–944. doi: 10.1212/WNL.34.7.939

Meltzer, C. C., Smith, G., DeKosky, S. T., Pollock, B. G., Mathis, C. A., Moore, R. Y., et al. (1998). Serotonin in aging, late-life depression, and Alzheimer's disease: the emerging role of functional imaging. *Neuropsychopharmacology* 18, 407–430. doi: 10.1016/S0893-133X(97)00194-2

Milgram, N. W., Adams, B., Callahan, H., Head, E., Mackay, W., Thirlwell, C., et al. (1999). Landmark discrimination learning in the dog. *Learn. Mem.* 6, 54–61.

Milgram, N. W., Head, E., Muggenburg, B. A., Holowachuk, D., Murphey, H., Estrada, J., et al. (2002a). Landmark discrimination learning in the dog: effects of age, an antioxidant fortified diet, and cognitive strategy. *Neurosci. Biobehav. Rev.* 26, 679–695. doi: 10.1016/S0149-7634(02)00039-8

Milgram, N. W., Zicker, S. C., Head, E., Muggenburg, B. A., Murphey, H., Ikeda-Douglas, C. J., et al. (2002b). Dietary enrichment counteracts age-associated cognitive dysfunction in canines. *Neurobiol. Aging* 23, 737–745. doi: 10.1016/S0197-4580(02)00020-9

Milgram, N. W., Head, E., Weiner, E., and Thomas, E. (1994). Cognitive functions and aging in the dog: acquisition of nonspatial visual tasks. *Behav. Neurosci.* 108, 57–68. doi: 10.1037/0735-7044.108.1.57

Milgram, N. W., Head, E., Zicker, S. C., Ikeda-Douglas, C., Murphey, H., Muggenberg, B. A., et al. (2004). Long-term treatment with antioxidants and a program of behavioral enrichment reduces age-dependent impairment in discrimination and reversal learning in beagle dogs. *Exp. Gerontol.* 39, 753–765. doi: 10.1016/j.exger.2004.01.007

Milgram, N. W., Head, E., Zicker, S. C., Ikeda-Douglas, C. J., Murphey, H., Muggenburg, B., et al. (2005). Learning ability in aged beagle dogs is preserved by behavioral enrichment and dietary fortification: a two-year longitudinal study. *Neurobiol. Aging* 26, 77–90. doi: 10.1016/j.neurobiolaging.2004.02.014

Mirra, S. S. (1997). The CERAD neuropathology protocol and consensus recommendations for the postmortem diagnosis of Alzheimer's disease: a commentary. *Neurobiol. Aging.* 18(Suppl. 4), S91–S94. doi: 10.1016/S0197-4580(97)00058-4

Montine, T. J., Neely, M. D., Quinn, J. F., Beal, M. F., Markesbery, W. R., Roberts, L. J., et al. (2002). Lipid peroxidation in aging brain and Alzheimer's disease. *Free Radic. Biol. Med.* 33, 620–626. doi: 10.1016/S0891-5849(02)00807-9

Morris, J. C., Storandt, M., McKeel, D. W. Jr., Rubin, E. H., Price, J. L., Grant, E. A., et al. (1996). Cerebral amyloid deposition and diffuse plaques in "normal" aging: evidence for presymptomatic and very mild Alzheimer's disease. *Neurology* 46, 707–719. doi: 10.1212/WNL.46.3.707

Morys, J., Narkiewicz, O., Maciejewska, B., Wegiel, J., and Wisniewski, H. M. (1994). Amyloid deposits and loss of neurones in the claustrum of the aged dog. *Neuroreport* 5, 1825–1828. doi: 10.1097/00001756-199409080-00035

Mullane, K., and Williams, M. (2013). Alzheimer's therapeutics: continued clinical failures question the validity of the amyloid hypothesis-but what lies beyond? *Biochem. Pharmacol.* 85, 289–305. doi: 10.1016/j.bcp.2012.11.014

Murphy, M. P., Morales, J., Beckett, T. L., Astarita, G., Piomelli, D., Weidner, A., et al. (2010). Changes in cognition and amyloid-β processing with long term cholesterol reduction using atorvastatin in aged dogs. *J. Alzheimers Dis.* 22, 135–150. doi: 10.3233/JAD-2010-100639

Nicoll, J. A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R. O. (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. *Nat. Med.* 9, 448–452. doi: 10.1038/nm840

Nicoll, J. A., Yamada, M., Frackowiak, J., Mazur-Kolecka, B., and Weller, R. O. (2004). Cerebral amyloid angiopathy plays a direct role in the pathogenesis of Alzheimer's disease. Pro-CAA position statement. *Neurobiol. Aging* 25, 589–597; discussion 603–604. doi: 10.1016/j.neurobiolaging.2004.02.003

Opii, W. O., Joshi, G., Head, E., Milgram, N. W., Muggenburg, B. A., Klein, J. B., et al. (2008). Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: relevance to Alzheimer's disease. *Neurobiol. Aging* 29, 51–70. doi: 10.1016/j.neurobiolaging.2006.09.012

Overk, C. R., and Masliah, E. (2014). Pathogenesis of synaptic degeneration in Alzheimer's disease and Lewy body disease. *Biochem. Pharmacol.* doi: 10.1016/j.bcp.2014.01.015

Pan, Y., Larson, B., Araujo, J. A., Lau, W., de Rivera, C., Santana, R., et al. (2010). Dietary supplementation with medium-chain TAG has long-lasting cognition-enhancing effects in aged dogs. *Br. J. Nutr.* 103, 1746–1754. doi: 10.1017/S0007114510000097

Papaioannou, N., Tooten, P. C. J., van Ederen, A. M., Bohl, J. R. E., Rofina, J., Tsangaris, T., et al. (2001). Immunohistochemical investigation of the brain of aged dogs. I. Detection of neurofibrillary tangles and of 4-hydroxynonenal protein, an oxidative damage product, in senile plaques. *Amyloid* 8, 11–21. doi: 10.3109/13506120108993810

Parker, H. G., Kim, L. V., Sutter, N. B., Carlson, S., Lorentzen, T. D., Malek, T. B., et al. (2004). Genetic structure of the purebred domestic dog. *Science* 304, 1160–1164. doi: 10.1126/science.1097406

Peremans, K., Audenaert, K., Blanckaert, P., Jacobs, F., Coopman, F., Verschooten, F., et al. (2002). Effects of aging on brain perfusion and serotonin-2A receptor binding in the normal canine brain measured with single photon emission tomography. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 26, 1393–1404. doi: 10.1016/S0278-5846(02)00306-8

Petanceska, S. S., DeRosa, S., Olm, V., Diaz, N., Sharma, A., Thomas-Bryant, T., et al. (2002). Statin therapy for Alzheimer's disease: will it work? *J. Mol. Neurosci.* 19, 155–161. doi: 10.1007/s12031-002-0026-2

Pohl, W. (1973). Dissociation of spatial discrimination deficits following frontal and parietal lesions in monkeys. *J. Comp. Physiol. Psychol.* 82, 227–239. doi: 10.1037/h0033922

Pop, V., Head, E., Berchtold, N. C., Glabe, C. G., Studzinski, C. M., Weidner, A. M., et al. (2010a). Aβ aggregation profiles and shifts in APP processing favor amyloidogenesis in canines. *Neurobiol. Aging* 33, 108–120. doi: 10.1016/j.neurobiolaging.2010.02.008

Pop, V., Head, E., Hill, M. A., Gillen, D., Berchtold, N. C., Muggenburg, B. A., et al. (2010b). Synergistic effects of long-term antioxidant diet and behavioral enrichment on β-amyloid load and non-amyloidogenic processing in aged canines. *J. Neurosci.* 30, 9831–9839. doi: 10.1523/JNEUROSCI.6194-09.2010

Pratico, D., Clark, C. M., Liun, F., Lee, V. Y.-M., and Trojanowski, J. Q. (2002). Increase of brain oxidative stress in mild cognitive impairment. *Arch. Neurol.* 59, 972–976. doi: 10.1001/archneur.59.6.972

Price, J. L., Ko, A. I., Wade, M. J., Tsou, S. K., McKeel, D. W., and Morris, J. C. (2001). Neuron number in the entorhinal cortex and CA1 in preclinical Alzheimer disease. *Arch. Neurol.* 58, 1395–1402. doi: 10.1001/archneur.58.9.1395

Prior, R., D'Urso, D., Frank, R., Prikulis, I., and Pavlakovic, G. (1996). Loss of vessel wall viability in cerebral amyloid angiopathy. *Neuroreport* 7, 562. doi: 10.1097/00001756-199601310-00044

Pugliese, M., Carrasco, J. L., Geloso, M. C., Mascort, J., Michetti, F., and Mahy, N. (2004). Gamma-aminobutyric acidergic interneuron vulnerability to aging in canine prefrontal cortex. *J. Neurosci. Res.* 77, 913–920. doi: 10.1002/jnr.20223

Pugliese, M., Gangitano, C., Ceccariglia, S., Carrasco, J. L., Del Fa, A., Rodriguez, M. J., et al. (2007). Canine cognitive dysfunction and the cerebellum: acetylcholinesterase reduction, neuronal and glial changes. *Brain Res.* 1139, 85–94. doi: 10.1016/j.brainres.2006.12.090

Pugliese, M., Geloso, M. C., Carrasco, J. L., Mascort, J., Michetti, F., and Mahy, N. (2006a). Canine cognitive deficit correlates with diffuse plaque maturation and S100β (−) astrocytosis but not with insulin cerebrospinal fluid level. *Acta Neuropathol.* 111, 519–528. doi: 10.1007/s00401-006-0052-1

Pugliese, M., Mascort, J., Mahy, N., and Ferrer, I. (2006b). Diffuse β-amyloid plaques and hyperphosphorylated tau are unrelated processes in aged dogs with behavioral deficits. *Acta Neuropathol.* 112, 175–183. doi: 10.1007/s00401-006-0087-3

Rapp, P. R. (1990). Visual discrimination and reversal learning in the aged monkey (*Macaca mulatta*). *Behav. Neurosci.* 6, 876–884. doi: 10.1037/0735-7044.104.6.876

Raz, N., Gunning-Dixon, F. M., Head, D., Dupuis, J. H., and Acker, J. D. (1998). Neuroanatomical correlates of cognitive aging: evidence from structural magnetic resonance imaging. *Neuropsychology* 12, 95–114. doi: 10.1037/0894-4105.12.1.95

Rensink, A. A., de Waal, R. M., Kremer, B., and Verbeek, M. M. (2003). Pathogenesis of cerebral amyloid angiopathy. *Brain Res. Brain Res. Rev.* 43, 207–223. doi: 10.1016/j.brainresrev.2003.08.001

Rissman, R. A., De Blas, A. L., and Armstrong, D. M. (2007). $GABA_A$ receptors in aging and Alzheimer's disease. *J. Neurochem.* 103, 1285–1292. doi: 10.1111/j.1471-4159.2007.04832.x

Rockwood, K., Kirkland, S., Hogan, D. B., MacKnight, C., Merry, H., Verreault, R., et al. (2002). Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch. Neurol.* 59, 223–227. doi: 10.1001/archneur.59.2.223

Rodriguez, E. G., Dodge, H. H., Birzescu, M. A., Stoehr, G. P., and Ganguli, M. (2002). Use of lipid-lowering drugs in older adults with and without dementia: a community-based epidemiological study. *J. Am. Geriatr. Soc.* 50, 1852–1856. doi: 10.1046/j.1532-5415.2002.50515.x

Rofina, J. E., Singh, K., Skoumalova-Vesela, A., van Ederen, A. M., van Asten, A. J., Wilhelm, J., et al. (2004). Histochemical accumulation of oxidative damage products is associated with Alzheimer-like pathology in the canine. *Amyloid* 11, 90–100. doi: 10.1080/13506120412331285779

Rofina, J. E., van Ederen, A. M., Toussaint, M. J., Secreve, M., van der Spek, A., van der Meer, I., et al. (2006). Cognitive disturbances in old dogs suffering from the canine counterpart of Alzheimer's disease. *Brain Res.* 1069, 216–226. doi: 10.1016/j.brainres.2005.11.021

Russell, M. J., Bobik, M., White, R. G., Hou, Y., Benjamin, S. A., and Geddes, J. W. (1996). Age-specific onset of β-amyloid in beagle brains. *Neurobiol. Aging* 17, 269–273. doi: 10.1016/0197-4580(95)02072-1

Russell, M. J., White, R., Patel, E., Markesbery, W. R., Watson, C. R., and Geddes, J. W. (1992). Familial influence on plaque formation in the beagle brain. *Neuroreport* 3, 1093–1096. doi: 10.1097/00001756-199212000-00015

Sarasa, L., Allue, J. A., Pesini, P., Gonzalez-Martinez, A., and Sarasa, M. (2013). Identification of β-amyloid species in canine cerebrospinal fluid by mass spectrometry. *Neurobiol. Aging* 34, 2125–2132. doi: 10.1016/j.neurobiolaging.2013.03.009

Satou, T., Cummings, B. J., Head, E., Nielson, K. A., Hahn, F. F., Milgram, N. W., et al. (1997). The progression of β-amyloid deposition in the frontal cortex of the aged canine. *Brain Res.* 774, 35–43. doi: 10.1016/S0006-8993(97)81684-8

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., et al. (1999). Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400, 173–177. doi: 10.1038/22124

Schliebs, R., and Arendt, T. (2006). The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. *J. Neural Transm.* 113, 1625–1644. doi: 10.1007/s00702-006-0579-2

Selkoe, D. J. (2008). Soluble oligomers of the amyloid β-protein impair synaptic plasticity and behavior. *Behav. Brain Res.* 192, 106–113. doi: 10.1016/j.bbr.2008.02.016

Selkoe, D. J., Bell, D. S., Podlisny, M. B., Price, D. L., and Cork, L. C. (1987). Conservation of brain amyloid proteins in aged mammals and humans with Alzheimer's disease. *Science* 235, 873–877. doi: 10.1126/science.3544219

Simic, G., Kostovic, I., Winblad, B., and Bogdanovic, N. (1997). Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. *J. Comp. Neurol.* 379, 482–494. doi: 10.1002/(SICI)1096-9861(19970324)379:4<482::AID-CNE2>3.0.CO;2-Z

Simons, M., Keller, P., Strooper, B. D., Beyreuther, K., Dotti, C. G., and Simons, K. (1998). Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6460–6464. doi: 10.1073/pnas.95.11.6460

Simons, M., Schwarzler, F., Lutjohann, D., von Bergmann, K., Beyreuther, K., Dichgans, J., et al. (2002). Treatment with simvastatin in normocholesterolemic patients with Alzheimer's disease: a 26-week randomized, placebo-controlled, double-blind trial. *Ann. Neurol.* 52, 346–350. doi: 10.1002/ana.10292

Siwak, C. T., Gruet, P., Woehrle, F., Schneider, M., Muggenburg, B. A., Murphey, H. L., et al. (2000). Behavioral activating effects of adrafinil in aged canines. *Pharmacol. Biochem. Behav.* 66, 293–300. doi: 10.1016/S0091-3057(00)00188-X

Siwak, C. T., Tapp, P. D., Head, E., Zicker, S. C., Murphey, H. L., Muggenburg, B. A., et al. (2005). Chronic antioxidant and mitochondrial cofactor administration improves discrimination learning in aged but not young dogs. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 461–469. doi: 10.1016/j.pnpbp.2004.12.011

Siwak, C. T., Tapp, P. D., and Milgram, N. W. (2001). Effect of age and level of cognitive function on spontaneous and exploratory behaviors in the beagle dog. *Learn. Mem.* 8, 317–325. doi: 10.1101/lm.41701

Siwak-Tapp, C. T., Head, E., Muggenburg, B. A., Milgram, N. W., and Cotman, C. W. (2007). Neurogenesis decreases with age in the canine hippocampus and correlates with cognitive function. *Neurobiol. Learn. Mem.* 88, 249–259. doi: 10.1016/j.nlm.2007.05.001

Siwak-Tapp, C. T., Head, E., Muggenburg, B. A., Milgram, N. W., and Cotman, C. W. (2008). Region specific neuron loss in the aged canine hippocampus is reduced by enrichment. *Neurobiol. Aging* 29, 39–50. doi: 10.1016/j.neurobiolaging.2006.09.018

Skoumalova, A., Rofina, J., Schwippelova, Z., Gruys, E., and Wilhelm, J. (2003). The role of free radicals in canine counterpart of senile dementia of the Alzheimer type. *Exp. Gerontol.* 38, 711–719. doi: 10.1016/S0531-5565(03)00071-8

Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A., et al. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* 88, 10540–10543. doi: 10.1073/pnas.88.23.10540

Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K., and Perry, G. (2000). Oxidative stress in Alzheimer's disease. *Biochim. Biophys. Acta* 1502, 139–144. doi: 10.1016/S0925-4439(00)00040-5

Smith, M. A., Sayre, L. M., Monnier, V. M., and Perry, G. (1996). Oxidative post-translational modifications in Alzheimer's disease. *Mol. Chem. Neuropathol.* 28, 41–48. doi: 10.1007/BF02815203

Sparks, D. L., Connor, D. J., Sabbagh, M. N., Petersen, R. B., Lopez, J., and Browne, P. (2006a). Circulating cholesterol levels, apolipoprotein E genotype and dementia severity influence the benefit of atorvastatin treatment in Alzheimer's disease: results of the Alzheimer's Disease Cholesterol-Lowering Treatment (ADCLT) trial. *Acta Neurol. Scand. Suppl.* 185, 3–7. doi: 10.1111/j.1600-0404.2006.00690.x

Sparks, D. L., Sabbagh, M., Connor, D., Soares, H., Lopez, J., Stankovic, G., et al. (2006b). Statin therapy in Alzheimer's disease. *Acta Neurol. Scand. Suppl.* 185, 78–86. doi: 10.1111/j.1600-0404.2006.00689.x

Sparks, D. L., Sabbagh, M. N., Connor, D. J., Lopez, J., Launer, L. J., Browne, P., et al. (2005a). Atorvastatin for the treatment of mild to moderate Alzheimer disease: preliminary results. *Arch. Neurol.* 62, 753–757. doi: 10.1001/archneur.62.5.753

Sparks, D. L., Sabbagh, M. N., Connor, D. J., Lopez, J., Launer, L. J., Petanceska, S., et al. (2005b). Atorvastatin therapy lowers circulating cholesterol but not free radical activity in advance of identifiable clinical benefit in the treatment of mild-to-moderate AD. *Curr. Alzheimer Res.* 2, 343–353. doi: 10.2174/1567205054367900

Studzinski, C. M., Christie, L. A., Araujo, J. A., Burnham, W. M., Head, E., Cotman, C. W., et al. (2006). Visuospatial function in the beagle dog: an early marker of cognitive decline in a model of human aging and dementia. *Neurobiol. Learn. Mem.* 86, 197–204. doi: 10.1016/j.nlm.2006.02.005

Su, M.-Y., Head, E., Brooks, W. M., Wang, Z., Muggenberg, B. A., Adam, G. E., et al. (1998). MR Imaging of anatomic and vascular characteristics in a canine model of human aging. *Neurobiol. Aging* 19, 479–485. doi: 10.1016/S0197-4580(98)00081-5

Su, M. Y., Tapp, P. D., Vu, L., Chen, Y. F., Chu, Y., Muggenburg, B., et al. (2005). A longitudinal study of brain morphometrics using serial magnetic resonance imaging analysis in a canine model of aging. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 389–397. doi: 10.1016/j.pnpbp.2004.12.005

Tapp, D., Siwak, C. T., Zicker, S. C., Head, E., Muggenburg, B. A., Cotman, C. W., et al. (2003a). An antioxidant enriched diet improves concept learning in aged dogs. *Soc. Neurosci. Abstr.* 836, 12.

Tapp, P. D., Siwak, C. T., Estrada, J., Head, E., Muggenburg, B. A., Cotman, C. W., et al. (2003b). Size and reversal learning in the beagle dog as a measure of executive function and inhibitory control in aging. *Learn. Mem.* 10, 64–73. doi: 10.1101/lm.54403

Tapp, P. D., Siwak, C. T., Gao, F. Q., Chiou, J. Y., Black, S. E., Head, E., et al. (2004a). Frontal lobe volume, function, and β-amyloid pathology in a canine model of aging. *J. Neurosci.* 24, 8205–8213. doi: 10.1523/JNEUROSCI.1339-04.2004

Tapp, P. D., Siwak, C. T., Head, E., Cotman, C. W., Murphey, H., Muggenburg, B. A., et al. (2004b). Concept abstraction in the aging dog: development of a protocol using successive discrimination and size concept tasks. *Behav. Brain Res.* 153, 199–210. doi: 10.1016/j.bbr.2003.12.003

Thal, D. R., Rub, U., Orantes, M., and Braak, H. (2002). Phases of A β-deposition in the human brain and its relevance for the development of, AD. *Neurology* 58, 1791–1800. doi: 10.1212/WNL.58.12.1791

Thelen, K. M., Rentsch, K. M., Gutteck, U., Heverin, M., Olin, M., Andersson, U., et al. (2006). Brain cholesterol synthesis in mice is affected by high dose of simvastatin but not of pravastatin. *J. Pharmacol. Exp. Ther.* 316, 1146–1152. doi: 10.1124/jpet.105.094136

Todd, P. A., and Goa, K. L. (1990). Simvastatin. a review of its pharmacological properties and therapeutic potential in hypercholesterolaemia. *Drugs* 40, 583–607. doi: 10.2165/00003495-199040040-00007

Tomic, J. L., Pensalfini, A., Head, E., and Glabe, C. G. (2009). Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction. *Neurobiol. Dis.* 35, 352–358. doi: 10.1016/j.nbd.2009.05.024

Torp, R., Head, E., and Cotman, C. W. (2000a). Ultrastructural analyses of β-amyloid in the aged dog brain: neuronal β-amyloid is localized to the plasma membrane. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 24, 801–810. doi: 10.1016/S0278-5846(00)00107-X

Torp, R., Head, E., Milgram, N. W., Hahn, F., Ottersen, O. P., and Cotman, C. W. (2000b). Ultrastructural evidence of fibrillar β-amyloid associated with neuronal membranes in behaviorally characterized aged dog brains. *Neuroscience* 93, 495–506. doi: 10.1016/S0306-4522(99)00568-0

Uchida, K., Miyauchi, Y., Nakayama, H., and Goto, N. (1990). Amyloid angiopathy with cerebral hemorrhage and senile plaque in aged dogs. *Nihon Juigaku Zasshi* 52, 605–611. doi: 10.1292/jvms1939.52.605

Uchida, K., Nakayama, H., and Goto, N. (1991). Pathological studies on cerebral amyloid angiopathy, senile plaques and amyloid deposition in visceral organs in aged dogs. *J. Vet. Med. Sci.* 53, 1037–1042. doi: 10.1292/jvms.53.1037

Uchida, K., Tani, Y., Uetsuka, K., Nakayama, H., and Goto, N. (1992). Immunohistochemical studies on canine cerebral amyloid angiopathy and senile plaques. *J. Vet. Med. Sci.* 54, 659–667. doi: 10.1292/jvms.54.659

Walker, L. C., Kitt, C. A., Struble, R. J., Wagster, M. V., Price, D. L., and Cork, L. C. (1988). The neural basis of memory decline in aged monkeys. *Neurobiol. Aging* 9, 657–666. doi: 10.1016/S0197-4580(88)80130-1

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Rowan, M. J., and Selkoe, D. J. (2002). Amyloid-β oligomers: their production, toxicity and therapeutic inhibition. *Biochem. Soc. Trans.* 30, 552–557. doi: 10.1042/BST0300552

Warren, J. M. (1964). "The behavior of carnivores and primates with lesions in the prefrontal cortex," in *The Frontal Granular Cortex and Behavior*, eds J. M. Warren and K. Akert (New York: McGraw-Hill Book Company), 168–191.

Wegiel, J., Wisniewski, H. M., and Soltysiak, Z. (1998). Region- and cell-type-specific pattern of tau phosphorylation in dog brain. *Brain Res.* 802, 259–266. doi: 10.1016/S0006-8993(98)00542-3

West, M. J. (1993). Regionally specific loss of neurons in the aging human hippocampus. *Neurobiol. Aging* 14, 287–293. doi: 10.1016/0197-4580(93)90113-P

West, M. J., Coleman, P. D., Flood, D. G., and Troncoso, J. C. (1994). Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. *Lancet* 344, 769–772. doi: 10.1016/S0140-6736(94)92338-8

West, M. J., Kawas, C. H., Martin, L. J., and Troncoso, J. C. (2000). The CA1 region of the human hippocampus is a hot spot in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 908, 255–259. doi: 10.1111/j.1749-6632.2000.tb06652.x

Wisniewski, H. M., Johnson, A. B., Raine, C. S., Kay, W. J., and Terry, R. D. (1970). Senile plaques and cerebral amyloidosis in aged dogs. *Lab. Investig.* 23, 287–296.

Wisniewski, H. M., Wegiel, J., Morys, J., Bancher, C., Soltysiak, Z., and Kim, K. S. (1990). "Aged dogs: an animal model to study β-protein amyloidogenesis," in *Alzheimer's Disease Epidemiology, Neuropathology: Neurochemistry and Clinics*, eds K. Maurer, R. Riederer, and H. Beckman (New York: Springer-Verlag), 151–167. doi: 10.1007/978-3-7091-3396-5_15

Wisniewski, T., Lalowski, M., Bobik, M., Russell, M., Strosznajder, J., and Frangione, B. (1996). Amyloid β 1-42 deposits do not lead to Alzheimer's neuritic plaques in aged dogs. *Biochem. J.* 313, 575–580.

Wolozin, B., Kellman, W., Ruosseau, P., Celesia, G. G., and Siegel, G. (2000). Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme a reductase inhibitors. *Arch. Neurol.* 57, 1439–1443. doi: 10.1001/archneur.57.10.1439

Wolozin, B., Wang, S. W., Li, N. C., Lee, A., Lee, T. A., and Kazis, L. E. (2007). Simvastatin is associated with a reduced incidence of dementia and Parkinson's disease. *BMC Med.* 5:20. doi: 10.1186/1741-7015-5-20

Zamrini, E., McGwin, G., and Roseman, J. M. (2004). Association between statin use and Alzheimer's disease. *Neuroepidemiology* 23, 94–98. doi: 10.1159/000073981

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Age-related changes of protein SUMOylation balance in the AβPP Tg2576 mouse model of Alzheimer's disease

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Alzheimer's disease (AD) is a complex disorder that affects the central nervous system causing a severe neurodegeneration. This pathology affects an increasing number of people worldwide due to the overall aging of the human population. In recent years SUMO protein modification has emerged as a possible cellular mechanism involved in AD. Some of the proteins engaged in the physiopathological process of AD, like BACE1, GSK3-β tau, AβPP, and JNK, are in fact subject to protein SUMO modifications or interactions. Here, we have investigated the SUMO/deSUMOylation balance and SUMO-related proteins during the onset and progression of the pathology in the Tg2576 mouse model of AD. We examined four age-stages (1.5, 3, 6, 17 months old) and observed shows an increase in SUMO-1 protein conjugation at 3 and 6 months in transgenic mice with respect to WT in both cortex and hippocampus. Interestingly this is paralleled by increased expression levels of Ubc9 and SENP1 in both brain regions. At 6 months of age also the SUMO-1 mRNA resulted augmented. SUMO-2-ylation was surprisingly decreased in old transgenic mice and was unaltered in the other time windows. The fact that alterations in SUMO/deSUMOylation equilibrium occur from the early phases of AD suggests that global posttranslational modifications may play an important role in the mechanisms underlying disease pathogenesis, thus providing potential targets for pharmacological interventions.

Keywords: sumoylation, Tg2576, Alzheimer's disease, SENP1, Ubc9, SUMO-1, SUMO-2/3, neurodegeneration

INTRODUCTION

Alzheimer's disease (AD) is considered one of the most common and debilitating pathologies in the elderly. AD is a slowly progressive neurodegenerative disease that is characterized by impairment of memory and eventually by other symptoms (Heun et al., 2013).

Research indicates that the disease is associated with the production of oligomers of amyloid beta (Aβ) leading to progressive neuritic plaque deposition and hyperphosphorylation of microtubule protein tau with subsequent formation of neurofibrillary tangles (Tiraboschi et al., 2004).

An "oxidative stress hypothesis" for AD has been recently postulated (Markesbery, 1997; Di Domenico et al., 2011; Leitao et al., 2011), albeit it is still unclear whether oxidative stress represents a trigger mechanism to unbalance normal cell functions or might be rather the consequence of pathogenic events. SUMOylation is among the PTMs that has been recently linked to AD (Lee et al., 2013). In fact, SUMOylation induces critical changes on AD-associated proteins like microtubule-associated protein tau (MAPT), amyloid β precursor protein (AβPP) (Georgopoulou et al., 2001; Marcus and Schachter, 2011), c-Jun terminal kinase (JNK) (Feligioni et al., 2011; Sclip et al., 2013) and AMPA receptors (Jaafari et al., 2013), that play an important role in neuronal physiology (Pittaluga et al., 2005, 2006; Holman et al., 2007), therefore providing novel targets for therapeutic intervention. SUMO family includes at least three paralogs (SUMO-1 to -3) ubiquitously expressed in all organism tissues including the brain (Droescher et al., 2013). SUMOs target proteins through non-covalent or covalent interactions affecting their cellular localization, aggregation, metabolism and activity (Steffan et al., 2004; Martin et al., 2007; Feligioni et al., 2011; Krumova et al., 2011; Droescher et al., 2013). Protein SUMOylation has recently been recognized to play a fundamental role in oxidative stress (Bossis and Melchior, 2006; Feligioni et al., 2011; Leitao et al., 2011; Feligioni and Nisticò, 2013), in the regulation of glutamate release (Feligioni et al., 2009) and also in the modification of activity of several intracellular proteins like AβPP and tau (Dorval and Fraser, 2006, 2007; Zhang and Sarge, 2008). As a consequence, SUMO-mediated alterations in specific intracellular signaling pathways could promote AD pathogenesis. Altogether, these observations indicate that SUMOylation could play an important role in the onset of AD, though its precise contribution still remains elusive. Here we performed an age-related analysis on the expression levels of global protein

SUMOylation and SUMO-related enzymes in the Tg2576 mouse modeling AD. Importantly, we observe significant differences in SUMO/deSUMOylation balance at an early stage of the pathology.

MATERIALS AND METHODS

ETHICS STATEMENT

All experiments were done in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee on animal experiments of EBRI "Rita Levi-Montalcini" Foundation (Rome, Italy).

BRAIN TISSUE DISSECTION

Adult male and female Tg2576 (Brecht et al., 2005) mice were sacrificed by cervical dislocation and immediately after hippocampal and cortical brain regions were dissected on ice. Both Tg2576 and WT mice were processed in parallel and were used for the experiments at the different age stages (1.5, 3, 6, and 17 months). Once removed, brain tissues were immediately placed in a cryopreservation solution {containing 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane [Tris, final concentration (f.c.) 0.01 M]} and stored at −80°C until needed. The cortical and hippocampal tissues of Tg2576 and their wild-type (WT) littermates have been kindly provided by Lundbeck AS (Denmark).

PREPARATION OF LYSATE FROM BRAIN TISSUE

Around 200 μg of mice tissues were lysed in 300 μl of Lysis Buffer solution (LB) made up of 1% Triton X-100 (Serva, Germany), complete protease inhibitor cocktail solution (Serva, Germany), phosphatase inhibitor cocktail solution (Serva, Germany), 20 mM of NEM (Sigma–Aldrich) and the following components (mM): TRIS acetate, 20; sucrose, 0.27; EDTA, 1; EGTA, 1; Na Orthovanadate, 1; NaF, 50; Na Pyrophosphate, 5; Na β-glycerophosphate, 10; DTT, 1.

Samples were then kept for 30 min on ice to allow protein solubilization. Later a centrifugation step of 10 min at 12000 rpm was applied to the samples and the supernatant was collected and stored at −20°C until needed.

WESTERN BLOT

Protein concentrations for each sample were determined by Bradford assay and the samples were directly analyzed by immunoblotting following resuspension in Laemmli buffer.

Equal amount of proteins (∼15 μg for each condition) were resolved by 10% SDS-polyacrylamide gels and blotted onto PVDF membrane (Serva, Germany). The proteins blotted on the membrane were then blocked for 1 h at room temperature using Tris-buffered saline-Tween (t-TBS) (M) Tris, 0, 02; NaCl, 0, 15; Tween 20, 0, 1%) containing 5% skimmed milk.

Later the membranes were treated with specific antibodies and the incubation last for 12 h at 4°C with mild agitation. The primary antibody used for western blot analysis are: rabbit amyloid precursor protein (AβPP) 1:2000 (Sigma-Aldrich, USA), rabbit SUMO-1 1:1000 (Cell Signaling, USA), rabbit SUMO-2/3 (18H8) 1:1000 (Cell Signaling, USA), rabbit SENP1 1:500 (Thermo scientific, USA), mouse UBC9 (C12) 1:1000 (Santa Cruz Biotechnology, USA), mouse β-actin 1:30000 (Sigma-Aldrich, Italy).

Tris-buffer saline solution (TBS) with 0, 1% of Tween 20 was used to wash the membranes for 50 min of wash in t-TBS. Then the blots were incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (UCS Diagnostic), as needed.

After 50 min of washes in t-TBS bands immunoreactivity was detected by enhanced chemiluminescence (ECL; WESTAR, Cyanagen, Italy). For all experiments stripping procedure was applied when the control of loading, performed blotting for β-actin, was required (stripping buffer from SignaGen, USA).

For each time point, a western blot has been performed in which proteins from six samples for WT and Tg2576 have been separated and analyzed. Where possible, membranes have been stripped and re-blotted for different antibodies with the purpose of using same loading conditions.

RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted from brain cortex and hippocampus using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RNA yield and purity were determined by spectrophotometry absorption at 260 and 280 nm. To obtain cDNA, an equal amount of mRNA (1 μg) underwent to Reverse Transcription (Promega, Madison, WI). The resulting cDNA was stored at −20°C until used for the further analysis. Messenger RNA expression was quantitatively measured with quantitative (q) real time PCR using iCycler Bio-Rad. The reaction was performed in a 25 μl volume using SensiMix SYBR Green and Fluorescein kit (Bioline, London, UK). All the measures were performed in triplicate. The reaction conditions were as follows: 95°C for 10 min (Polymerase activation), followed by 40 cycles at 95°C for 15, 55–50°C (Temp. depends on the Tm of primers) for 15 s and 72°C for 15 s. The reaction mixture without the cDNA was used as control.

The primer sequences used in this study were as following for SUMO-1: forward 5′-GCCTGGGACATGGGTTT-3′ and reverse 5′-TTAATGAAGCTGGTACAGACGATG-3′; SUMO-2: forward 5′-GGCAGGGTTTGTCAATGAGGC-3′ and reverse 5′-CTGGAGTAAAGTA GTAGCAGGCTC-3′; SUMO-3: forward 5′-GAGGCAGGGCTTGTCAATGAG-3′ and reverse 5′-GGTC AGGACAACGGTTGGGTG-3′; SENP1: forward 5′-AATGGCTG ATGATGATGTG-3′ and reverse 5′-TTGGACAAGGATTAGA CTGAAT-3′; UBC9: forward 5′-CATCCAGCCTTCGT AAA CC-3′ and reverse 5′-GCTAACAGGCAGGGAGAT-3′; glyceraldehydes-3-phosphate dehydrogenase (GAPDH): forward 5′-GCCA AGGCTGTGGGCAAGGT-3′ and reverse 5′-TCTCCAGGCGGC ACGTCAGA-3′.

The *Ct* values of the specific gene of interest were normalized to the *Ct* value of the endogenous control, GAPDH, and the comparative *Ct* method ($2^{-\Delta\Delta Ct}$) was then applied using WT mice group as calibrator.

STATISTICAL ANALYSIS

In the western blots and real time PCR experiments, *T*-test analysis was performed and $p < 0.05$ was considered statistically significant. Statistical analysis for biochemical experiments

was performed using GraphPad PRISM 5. A number (as indicated in figure legends) of animal tissues has been used at the same time point for the experiments. Values shown represent the mean ± s.e.m. Final western blots have been run using representative sampling

RESULTS

AβPP INCREASES IN Tg2576 MICE

The Tg2576 transgenic mouse carries a transgene coding for the 695-amino acid isoform of human AβPP derived from a large Swedish family with early-onset AD (Hsiao et al., 1996). This mouse model of AD expresses high concentrations of mutant Aβ, develops a significant number of amyloid plaques, and displays functional deficits like decreased dendritic spine density, impaired long-term potentiation (LTP), and behavioral deficits (Jacobsen et al., 2006; Balducci et al., 2011).

In order to validate the mice model used in this work we measured AβPP immunoreactivity in the cortical and hippocampal tissues from the brains of Tg2576 and WT mice. Tg2576 are expected to have an augmented expression of AβPP because, beside the murine endogenous AβPP, they genetically over-express human mutated AβPP. Indeed, both cortex and hippocampus prepared from Tg2576 mice showed a more intense immunoreactivity of the AβPP band corresponding to 95–100 kDa compared to WT (**Figure 1D**).

PROTEIN SUMOylation CHANGES IN THE CORTEX OF Tg2576 MICE DURING ONTOGENESIS

The cerebral cortex is among the most vulnerable brain regions affected by the disease showing progressive plaque burden and tissue atrophy (Tosun et al., 2011). Although in a less severe manner, some biochemical hallmarks have also been reported in different AD mice models (Sclip et al., 2011; Izco et al., 2014).

In order to evaluate protein SUMOylation changes during disease progression, we dissected and lysated cortical tissues from 1.5, 3, 6, 17 months old Tg2576 and their age-matched WT littermates. Protein SUMOylation has been revealed by western blotting using an anti-SUMO-1 and an anti-SUMO-2/3 antibody. SUMO-1 and SUMO2/3 for each time point has been normalized against the actin. Interestingly, the ratio in protein SUMO-1-ylation between Tg2576 and WT mice is increased at early stages, and it later decreases following a bell-shaped curve. In fact, a peak of SUMO-1-ylation is observed at 3 and 6 months old animals (**Figure 1A**, 3 months: 1.65 ± 0.16 $p < 0.05$; 6 months: 1.78 ± 0.08 $p < 0.01$).

Noteworthy a protein SUMOylated band, absent in the other ages analyzed, appears at 6 months of age. The immunoreactivity of this band results more intense in the Tg2576 mice compared to controls (**Figure 1A**, black arrow). Further studies are required to identify which protein becomes target of SUMO-1 at this stage, and whether this protein modification has a role in AD pathology.

SUMO-2/3-ylation did not show any differences in the ratio between Tg2576 and WT mice at different ages except for 17 months old mice, where it resulted drastically decreased in transgenic mice (**Figure 1B**, 17 months: 0.35 ± 0.05 $p < 0.01$). Further experiments are required to understand what implication this reduction may have. As for the SUMO-1 western blot, also for SUMO-2/3 a more intense band is detectable (**Figure 1B**, black arrow).

PROTEIN SUMOylation CHANGES IN THE HIPPOCAMPUS OF Tg2576 MICE DURING ONTOGENESIS

The hippocampus is the part of the brain that is involved in memory formation, being one of the first regions to suffer damage in AD. Since Tg2576 show cognitive impairment caused by hippocampal dysfunction even before the onset of the pathology (D'Amelio et al., 2011), we analyzed protein SUMOylation changes between Tg2576 and WT mice also in this brain area.

Similarly to the cortex, SUMO-1-ylation is increased in the hippocampus of 3 and 6 months old Tg2576 compared to WT (**Figure 2A**, 3 months: 1.71 ± 0.07 $p < 0.01$; 6 months: 2.07 ± 0.39 $p < 0.01$).

SUMO-2/3-ylation did not show any differences in the ratio between Tg2576 and WT mice at different ages except for 17 months old, where surprisingly it resulted decreased in transgenic mice (**Figure 2B**, 17 months: 0.65 ± 0.01 $p < 0.01$). This result needs more investigation in order to understand its potential implication in the pathophysiology of AD.

SUMO PATHWAY RELATED PROTEINS IN Tg2576 TRANSGENIC MICE ONTOGENESIS

The protein conjugation by SUMO occurs through the activity of four key enzymes which form the SUMOylation pathway. Among them Ubc9 and SENP1 are the most important since they are dedicated exclusively to protein SUMOylation. On the one hand Ubc9 binds and transports SUMO to the target protein, on the other SENP1 is the isopeptidase that regulates the deSUMOylation (Droescher et al., 2013; Feligioni and Nisticò, 2013). Ubc9 and SENP1 are critical for the maintenance of SUMO/deSUMOylation balance, therefore their expression levels have been assessed by western blotting in cortical and hippocampal tissue from Tg2576 and WT mice. The expression level of the two proteins has been normalized on actin and the ratio between Tg2576 and WT has been analyzed.

Interestingly, we found that the expression level of Ubc9 is modulated both in cortical and hippocampal tissue. In fact, the western blots show for both WT and Tg2576 an increase of Ubc9 from 3 to 17 months with a peak at 6 months of age. Moreover Ubc9 seems to be highly expressed in Tg2576 compared to WT at different ages (**Figure 1C**, 6 months: 1.57 ± 0.16 $p < 0.05$) (**Figure 2C**, 3 months: 1.42 ± 0.24 $p < 0.05$; 6 months: 2.22 ± 0.37 $p < 0.01$). Conversely, immunoreactivity of SENP1 seems to be augmented in the cortex of transgenic mice at 3 and 6 months and only at 6 months in hippocampus of Tg2576 compared to age-matched WT animals (**Figure 1C**, 3 months: 1.75 ± 0.21 $p < 0.05$; 6 months: 2.51 ± 0.21 $p < 0.01$) (**Figure 2C**, 6 months: 2.15 ± 0.42 $p < 0.01$).

SUMO-1-, SUMO-2-, SUMO-3-, SENP1- AND Ubc9-mRNA IN BRAIN CORTEX AND HIPPOCAMPUS

SUMO-1 mRNA levels in the cortex (**Figure 3A**) and hippocampus (**Figure 4A**) of Tg2576 mice at 1.5 months of age were similar to the levels measured in WT mice, whereas at 6 months of age their levels were significantly higher than that of WT mice.

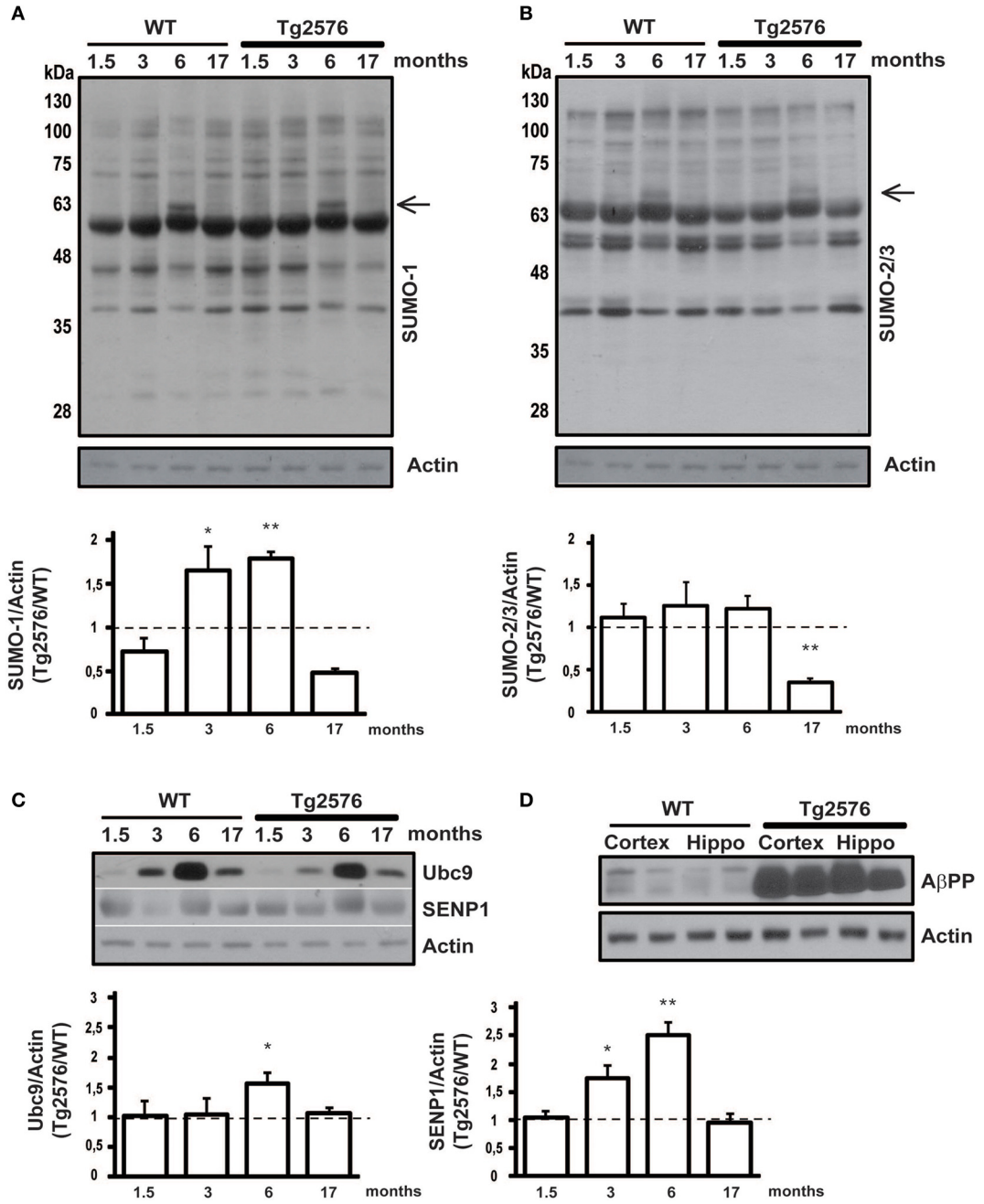

FIGURE 1 | Protein SUMOylation and SUMO-related proteins expression level in cortical tissue. Representative western blots of WT and Tg2576 mice cortical tissue lysate. Cortex of WT and Tg2576 animals was analyzed at four time points (1.5, 3, 6, and 17 months of age). Anti-SUMO-1 **(A)**, anti-SUMO-2/3 **(B)**, anti-Ubc9, anti-SENP1 **(C)**, and anti-AβPP **(D)** have been used for the protein detections. Results are expressed as ratio between Tg2576 and WT. Data represent means ± s.e.m. of 6 animals for each age. (*t*-test) $^{**}p < 0.01$ or $^{*}p < 0.05$ Tg2576 vs. WT. β-Actin was used as loading control.

On the contrary, we did not find any significant changes in SUMO-2, SUMO-3, SENP1, and Ubc9 mRNA levels in the cortex (**Figures 3B–E**) and hippocampus (**Figures 4B–E**) of Tg2576 and WT mice at the age of 1.5 and 6 months. The SUMO-1 mRNA increase is in accordance with the elevated level of SUMO-1-ylation that has been found in cortex and hippocampus at 6 months of age. It seems that SUMO-1 synthesis is augmented in order to be disposable for conjugation, whereas the Ubc9 and SENP1 protein levels are sufficient in both tissues to maintain SUMOylation pathway functional. The SUMO-2 and -3 mRNA level is in line with protein SUMO-2/3-ylation expression that resulted unchanged up to 6 months of age.

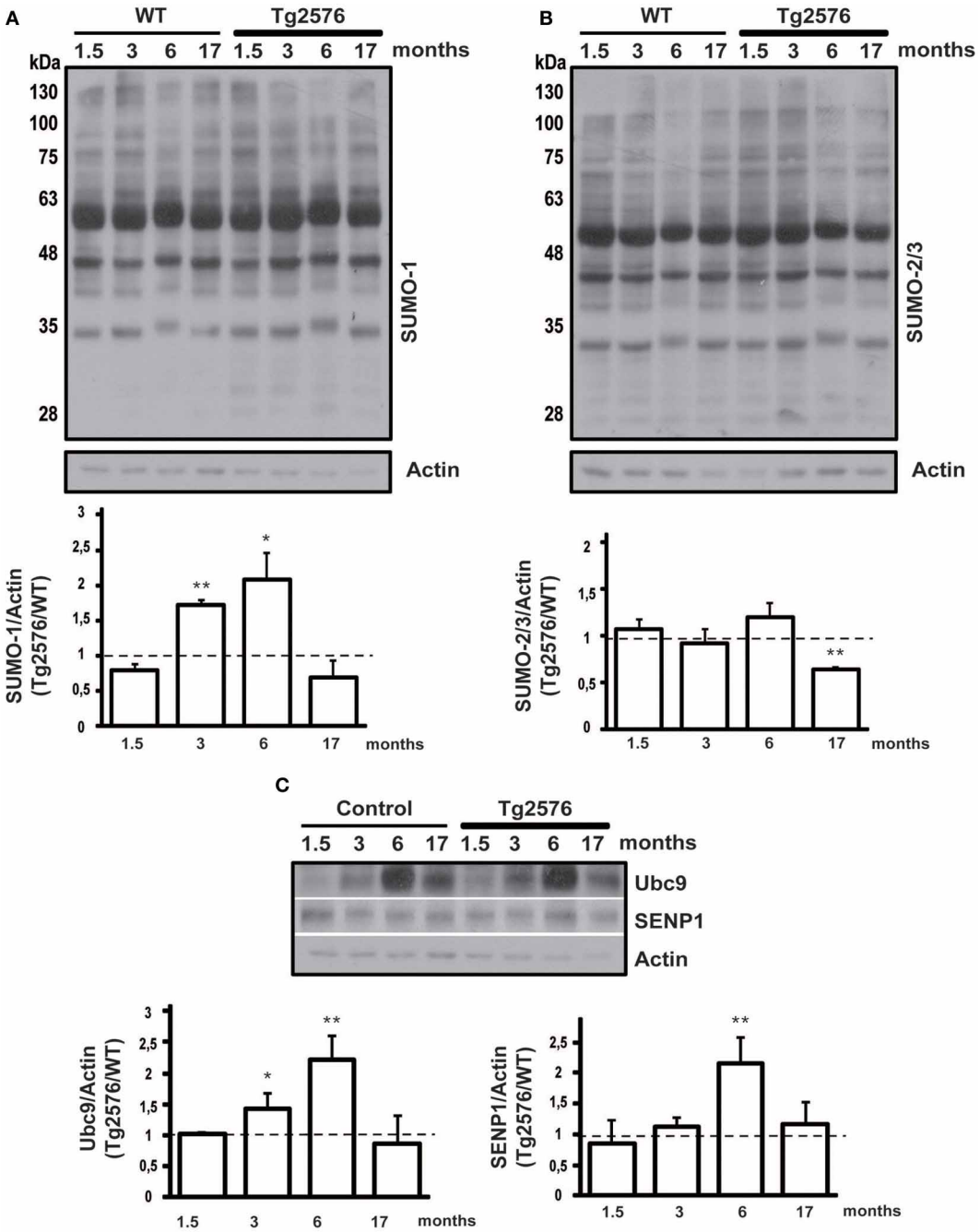

FIGURE 2 | Protein SUMOylation and SUMO-related proteins expression level in hippocampal tissue. Representative western blots of WT and Tg2576 mice hippocampal tissue lysate. Hippocampus of WT at four time points (1.5, 3, 6, and 17 months of age) was compared with their age-matched Tg2576 mice. Anti-SUMO-1 **(A)**, anti-SUMO-2/3 **(B)**, anti-Ubc9 and anti-SENP1 **(C)** have been used for protein detections. Results are expressed as ratio between Tg2576 and WT. Data represent means ± s.e.m. of 6 animals for each age. (*t*-test) $^{**}p < 0.01$ or $^{*}p < 0.05$ Tg2576 vs. WT. β-Actin was used as loading control.

DISCUSSION

Tg2576 mice model were developed to test the amyloid hypothesis of AD. These mice model answered partially the question whether AD pathology comes from the accumulation of Aβ species. Aβ species, although poorly detectable, can be found in the soluble form (monomers, dimers, trimmers or oligomers) already at a young age (<5 months old) (Kawarabayashi et al., 2001; Klingner et al., 2003; Lesné et al., 2006), but they reach a highly detectable level around 6–8 months (Hsiao et al., 1996). Insoluble amyloid plaques start to form at around 9–10 months in hippocampus and cortex (Hsiao et al., 1996). This model also shows the typical AD cognitive impairment with a mild onset at 3 months, which

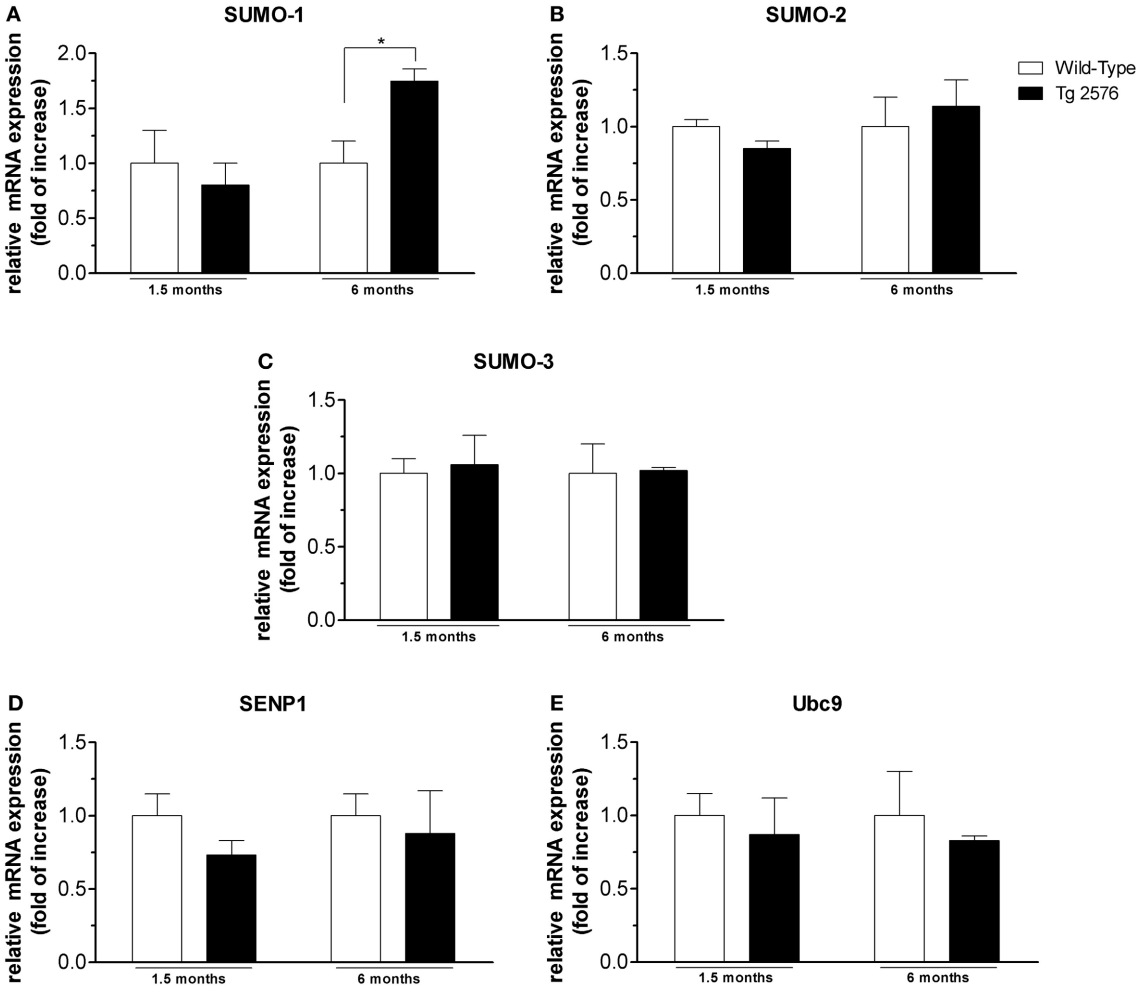

FIGURE 3 | mRNA expression level of SUMO and SUMO-related proteins in cortical tissues. Analyses of SUMO-1 **(A)**, SUMO-2 **(B)**, SUMO-3 **(C)**, SENP1 **(D)**, Ubc9 **(E)** mRNA expression in brain cortex of Tg2576 and WT mice at the age of 1.5 and 6 months. The mRNA expression levels, determined by real-time polymerase chain reaction (RT-PCR), were expressed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as fold increase relative to WT animals. Data represent mean ± s.e.m. of 4 animals. (*t*-test) $*p < 0.05$ Tg2576 vs. WT.

is exacerbated starting from 6 months of age. However, Tg2576 do not resemble all the features of AD since they do not display any formation of neurofibrillary tangles, marked neuronal loss or gross brain atrophy, (Irizarry et al., 1997), but are anyway a very useful model to study AD amyloid-related pathology (Deacon et al., 2008).

Several AD-associated proteins undergo SUMO protein modification, leading to hypothesize that SUMO plays a fundamental role in AD pathogenesis. Despite that, we still lack information about how SUMO/deSUMOylation process is altered during AD onset and development. Therefore a better understanding of SUMO changes during AD pathogenesis could help to disclose the role of protein modification in the onset of AD. Notably, McMillan and co-authors (McMillan et al., 2011) reported that no clear differences in global protein SUMOylation can be measured in adult Tg2576 (9 months old) vs. their respective WT.

In this manuscript, we decided to investigate SUMOylation changes during mice lifespan analyzing four time points. To our knowledge this is the first ontogenetic study of SUMO/deSUMOylation profile in an AD model.

Yun and co-authors have shown in *in-vitro* experiments that SUMO-1 can be considered a modulator of the synthesis of Aβ oligomers (Yun et al., 2013). In fact SUMO-1, but not SUMO-2 or -3, when overexpressed in neuronal or cell culture, increases BACE1 level which mediates the amiloydogenic cleavage of AβPP. Moreover, as previously reported in 18 months old AβPP transgenic mice (Yun et al., 2013) SUMO-1 free protein is increased in their cortical tissue. So, it has been postulated that the increase of Aβ accumulation together with an accrual of cell oxidative stress can act synergistically to enhance protein SUMO-1-ylation. By conjugating BACE1, SUMO-1 intensifies Aβ oligomers production (Yun et al., 2013). Our data show that already at the age of 3 months, protein SUMO-1-ylation is augmented both in cortical and hippocampal tissue of Tg2576 model compared to their respective WT.

Therefore it can be hypothesized that at early stages (3 months) the "cell stress" might contribute more to the increase

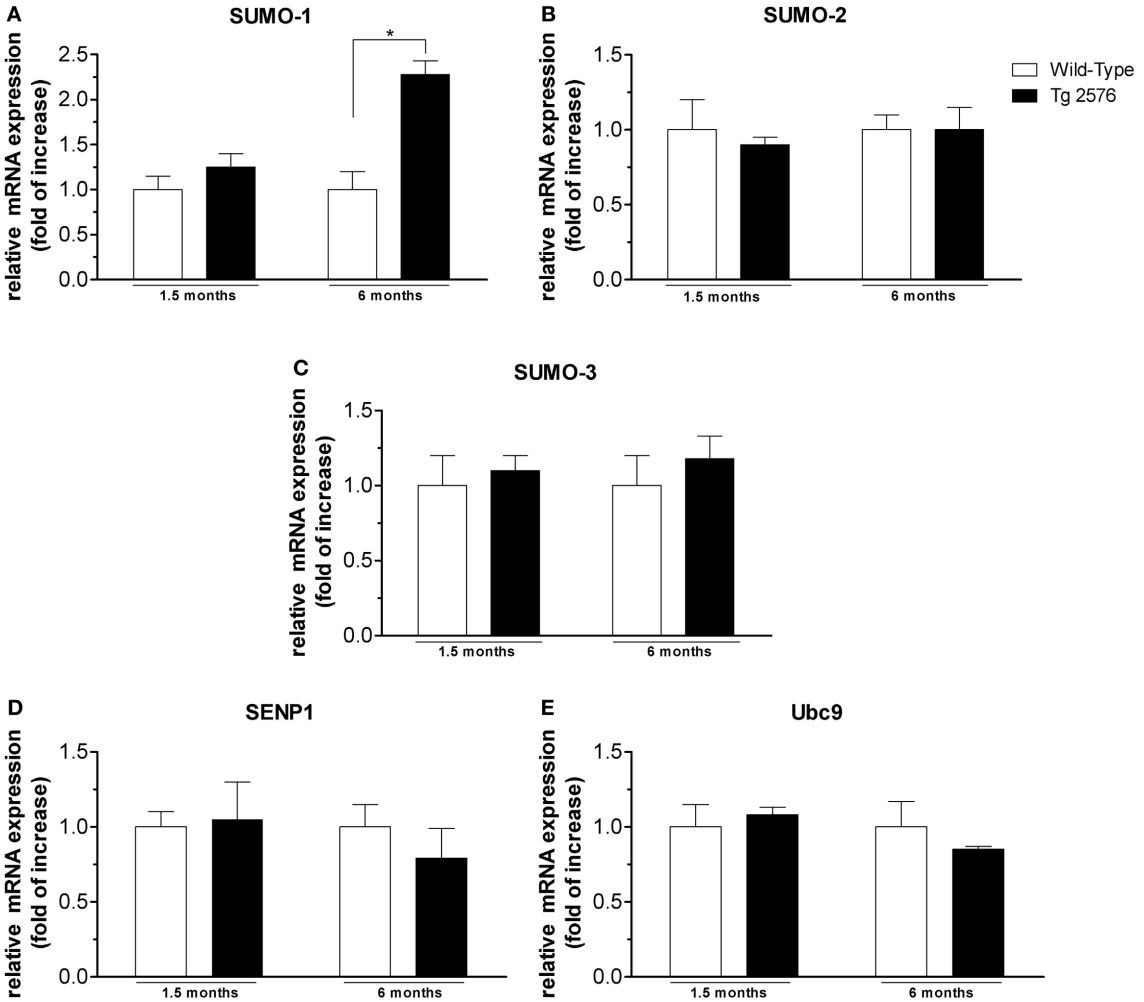

FIGURE 4 | mRNA expression level of SUMO and SUMO-related proteins in hippocampal tissues. Analyses of SUMO-1 **(A)**, SUMO-2 **(B)**, SUMO-3 **(C)**, SENP1 **(D)**, Ubc9 **(E)** mRNA expression in brain hippocampus of Tg2576 and WT mice at the age of 1.5 and 6 months. The mRNA expression levels, determined by real-time polymerase chain reaction (RT-PCR), were expressed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as fold increase relative to WT animals. Data represent mean ± s.e.m. of 4 animals. (*t*-test) $^*p < 0.05$ Tg2576 vs. WT.

in SUMO-1-ylation compared to Aβ oligomers which are present at low levels at this age. As a result, SUMO-1-ylation activates multiple signaling pathways, like BACE1, GSK3β, JNK activation, that can contribute to boost the production of Aβ oligomers. The feedback loop established between SUMO-1, Aβ oligomers and cell stress cooperate to the development of AD neuropathology. Interestingly, SUMO-2-ylation does not change between Tg2576 and WT except for 17 months old transgenic mice where it is drastically diminished both in cortex and hippocampus. This aspect also deserves a deeper investigation since very little is known about SUMO-2/3 contribution to AD.

Ubc9 and SENP1 are critical enzymes for protein SUMOylation (Droescher et al., 2013; Feligioni and Nisticò, 2013). An increase of SUMOylation is associated with the onset of several pathologies including cancer. For example, Ubc9 expression, the E2 enzyme which facilitates SUMOylation, has been reported increased in primary colon and prostate cancer compared with normal tissue (Moschos et al., 2010). We here demonstrate that the increase of SUMOylation in Tg2576 brain tissues corresponds to a high expression level of Ubc9 at the same time points. Therefore Ubc9 plays a fundamental role for SUMOylation event also in AD. On the other hand, the over-functionality of SUMOylation in Tg2576 is also supported by an elevated presence of the SUMO protease SENP1. Our results are in line with previous data where SENP-1 was found increased during oxygen-glucose deprivation (OGD) experiments, suggesting that the neuronal response could involve a complex interplay between SUMOylation and deSUMOylation (Cimarosti et al., 2012).

The analysis of mRNA expression has shown an increase of SUMO-1 RNA only at the age of 6 months both in cortex and hippocampus. This is in line with our western blot data where the major increase of SUMOylation was exactly found at 6 months of age in Tg2576 mice, where probably there is a need for more protein synthesis. In contrast, although we reported an increase in protein expression level of both Ubc9 and SENP1, the mRNA

expression of both proteins was unchanged. We can therefore speculate that the degradation system of Ubc9 and SENP1 could be decreased during lifespan of mice. Further studies should be carried out in order to better clarify this aspect. In line with a previous work (McMillan et al., 2011), our experiments did not show significant changes in protein SUMO-2/3-ylation in AD mice up to 6 months of age. SUMO-2/3 role in AD remains still elusive. However, in *in-vitro* experiments it has been shown that SUMO-2 increases N-terminal ADAM-cleaved AβPP fragment (α-NTF) while a mutant form of SUMO-2 lacking the SUMOylation activity secreted significantly more N-terminal BACE1-cleaved AβPP fragment (β-NTF) and Aβ (Li et al., 2003). In line with this report, we can speculate that the reduction in protein SUMO-2/3-ylation that was observed in 17 months old Tg2576 mice is concomitant with an elevated amyloid deposition which occurs at this stage in AD mice model (Jacobsen et al., 2006). In conclusion, here we report for the first time that protein SUMO/deSUMOylation equilibrium is unbalanced in a mouse model of AD at a very early stage of the pathology. We believe that this event can contribute to the onset of AD pathogenesis. Future investigation on the target(s) of protein SUMOylation at an early stage could possibly lead to the identification of novel pharmacological targets.

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REFERENCES

Balducci, C., Mehdawy, B., Mare, L., Giuliani, A., Lorenzini, L., Sivilia, S., et al. (2011). The γ-secretase modulator CHF5074 restores memory and hippocampal synaptic plasticity in plaque-free Tg2576 mice. *J. Alzheimer's Dis.* 24, 799–816. doi: 10.3233/JAD-2011-101839

Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol. Cell* 21, 349–357. doi: 10.1016/j.molcel.2005.12.019

Brecht, S., Kirchhof, R., Chromik, A., Willesen, M., Nicolaus, T., Raivich, G., et al. (2005). Specific pathophysiological functions of JNK isoforms in the brain. *Eur. J. Neurosci.* 21, 363–377. doi: 10.1111/j.1460-9568.2005.03857.x

Cimarosti, H., Ashikaga, E., Jaafari, N., Dearden, L., Rubin, P., Wilkinson, K. A., et al. (2012). Enhanced SUMOylation and SENP-1 protein levels following oxygen and glucose deprivation in neurones. *J. Cereb. Blood Flow Metab.* 32, 17–22. doi: 10.1038/jcbfm.2011.146

D'Amelio, M., Cavallucci, V., Middei, S., Marchetti, C., Pacioni, S., Ferri, A., et al. (2011). Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. *Nat. Neurosci.* 14, 69–76. doi: 10.1038/nn.2709

Deacon, R. M. J., Cholerton, L. L., Talbot, K., Nair-Roberts, R. G., Sanderson, D. J., Romberg, C., et al. (2008). Age-dependent and -independent behavioral deficits in Tg2576 mice. *Behav. Brain Res.* 189, 126–138. doi: 10.1016/j.bbr.2007.12.024

Di Domenico, F., Coccia, R., Butterfield, D. A., and Perluigi, M. (2011). Circulating biomarkers of protein oxidation for Alzheimer disease: expectations within limits. *Biochim. Biophys. Acta* 1814, 1785–1795. doi: 10.1016/j.bbapap.2011.10.001

Dorval, V., and Fraser, P. E. (2006). Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. *J. Biol. Chem.* 281, 9919–9924. doi: 10.1074/jbc.M510127200

Dorval, V., and Fraser, P. E. (2007). SUMO on the road to neurodegeneration. *Biochim. Biophys. Acta* 1773, 694–706. doi: 10.1016/j.bbamcr.2007.03.017

Droescher, M., Chaugule, V. K., and Pichler, A. (2013). SUMO rules: regulatory concepts and their implication in neurologic functions. *Neuromolecular Med.* 15, 639–660. doi: 10.1007/s12017-013-8258-6

Feligioni, M., Brambilla, E., Camassa, A., Sclip, A., Arnaboldi, A., Morelli, F., et al. (2011). Crosstalk between JNK and SUMO signaling pathways: deSUMOylation is protective against H(2)O(2)-induced cell injury. *PLoS ONE* 6:e28185. doi: 10.1371/journal.pone.0028185

Feligioni, M., Nishimune, A., and Henley, J. M. (2009). Protein SUMOylation modulates calcium influx and glutamate release from presynaptic terminals. *Eur. J. Neurosci.* 29, 1348–1356. doi: 10.1111/j.1460-9568.2009.06692.x

Feligioni, M., and Nisticò, R. (2013). SUMO: a (Oxidative) stressed protein. *Neuromolecular Med.* 15, 707–719. doi: 10.1007/s12017-013-8266-6

Georgopoulou, N., McLaughlin, M., McFarlane, I., and Breen, K. C. (2001). The role of post-translational modification in beta-amyloid precursor protein processing. *Biochem. Soc. Symp.* 23–36.

Heun, R., Schoepf, D., Potluri, R., and Natalwala, A. (2013). Alzheimer's disease and co-morbidity: increased prevalence and possible risk factors of excess mortality in a naturalistic 7-year follow-up. *Eur. Psychiatry?* 28, 40–48. doi: 10.1016/j.eurpsy.2011.06.001

Holman, D., Feligioni, M., and Henley, J. M. (2007). Differential redistribution of native AMPA receptor complexes following LTD induction in acute hippocampal slices. *Neuropharmacology* 52, 92–99. doi: 10.1016/j.neuropharm.2006.05.022

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., et al. (1996). Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274, 99–102. doi: 10.1126/science.274.5284.99

Irizarry, M. C., McNamara, M., Fedorchak, K., Hsiao, K., and Hyman, B. T. (1997). APPSw transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1. *J. Neuropathol. Exp. Neurol.* 56, 965–973. doi: 10.1097/00005072-199709000-00002

Izco, M., Martínez, P., Corrales, A., Fandos, N., García, S., Insua, D., et al. (2014). Changes in the brain and plasma Aβ peptide levels with age and its relationship with cognitive impairment in the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Neuroscience* 263C, 269–279. doi: 10.1016/j.neuroscience.2014.01.003

Jaafari, N., Konopacki, F. A., Owen, T. F., Kantamneni, S., Rubin, P., Craig, T. J., et al. (2013). SUMOylation is required for glycine-induced increases in AMPA receptor surface expression (ChemLTP) in hippocampal neurons. *PLoS ONE* 8:e52345. doi: 10.1371/journal.pone.0052345

Jacobsen, J. S., Wu, C., Redwine, J. M., Comery, T. A., Arias, R., Bowlby, M., et al. (2006). Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5161–5166. doi: 10.1073/pnas.0600948103

Kawarabayashi, T., Younkin, L. H., Saido, T. C., Shoji, M., Ashe, K. H., and Younkin, S. G. (2001). Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. *J. Neurosci.* 21, 372–381.

Klingner, M., Apelt, J., Kumar, A., Sorger, D., Sabri, O., Steinbach, J., et al. (2003). Alterations in cholinergic and non-cholinergic neurotransmitter receptor densities in transgenic Tg2576 mouse brain with beta-amyloid plaque pathology. *Int. J. Dev. Neurosci.* 21, 357–369. doi: 10.1016/j.ijdevneu.2003.08.001

Krumova, P., Meulmeester, E., Garrido, M., Tirard, M., Hsiao, H.-H., Bossis, G., et al. (2011). Sumoylation inhibits alpha-synuclein aggregation and toxicity. *J. Cell Biol.* 194, 49–60. doi: 10.1083/jcb.201010117

Lee, L., Sakurai, M., Matsuzaki, S., Arancio, O., and Fraser, P. (2013). SUMO and Alzheimer's disease. *Neuromolecular Med.* 15, 720–736. doi: 10.1007/s12017-013-8257-7

Leitao, B. B., Jones, M. C., and Brosens, J. J. (2011). The SUMO E3-ligase PIAS1 couples reactive oxygen species-dependent JNK activation to oxidative cell death. *FASEB J.* 25, 3416–3425. doi: 10.1096/fj.11-186346

Lesné, S., Koh, M. T., Kotilinek, L., Kayed, R., Glabe, C. G., Yang, A., et al. (2006). A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* 440, 352–357. doi: 10.1038/nature04533

Li, Y., Wang, H., Wang, S., Quon, D., Liu, Y.-W., and Cordell, B. (2003). Positive and negative regulation of APP amyloidogenesis by sumoylation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 259–264. doi: 10.1073/pnas.0235361100

Marcus, J. N., and Schachter, J. (2011). Targeting post-translational modifications on tau as a therapeutic strategy for Alzheimer's disease. *J. Neurogenet.* 25, 127–133. doi: 10.3109/01677063.2011.626471

Markesbery, W. R. (1997). Oxidative stress hypothesis in Alzheimer's disease. *Free Radic. Biol. Med.* 23, 134–147. doi: 10.1016/S0891-5849(96)00629-6

Martin, S., Nishimune, A., Mellor, J. R., and Henley, J. M. (2007). SUMOylation regulates kainate-receptor-mediated synaptic transmission. *Nature* 447, 321–325. doi: 10.1038/nature05736

McMillan, L. E., Brown, J. T., Henley, J. M., and Cimarosti, H. (2011). Profiles of SUMO and ubiquitin conjugation in an Alzheimer's disease model. *Neurosci. Lett.* 502, 201–208. doi: 10.1016/j.neulet.2011.07.045

Moschos, S. J., Jukic, D. M., Athanassiou, C., Bhargava, R., Dacic, S., Wang, X., et al. (2010). Expression analysis of Ubc9, the single small ubiquitin-like modifier (SUMO) E2 conjugating enzyme, in normal and malignant tissues. *Hum. Pathol.* 41, 1286–1298. doi: 10.1016/j.humpath.2010.02.007

Pittaluga, A., Feligioni, M., Longordo, F., Luccini, E., and Raiteri, M. (2006). Trafficking of presynaptic AMPA receptors mediating neurotransmitter release: neuronal selectivity and relationships with sensitivity to cyclothiazide. *Neuropharmacology* 50, 286–296. doi: 10.1016/j.neuropharm.2005.09.004

Pittaluga, A., Segantini, D., Feligioni, M., and Raiteri, M. (2005). Extracellular protons differentially potentiate the responses of native AMPA receptor subtypes regulating neurotransmitter release. *Br. J. Pharmacol.* 144, 293–299. doi: 10.1038/sj.bjp.0705960

Sclip, A., Antoniou, X., Colombo, A., Camici, G. G., Pozzi, L., Cardinetti, D., et al. (2011). c-Jun N-terminal kinase regulates soluble Aβ oligomers and cognitive impairment in AD mouse model. *J. Biol. Chem.* 286, 43871–43880. doi: 10.1074/jbc.M111.297515

Sclip, A., Arnaboldi, A., Colombo, I., Veglianese, P., Colombo, L., Messa, M., et al. (2013). Soluble Aβ oligomer-induced synaptopathy: c-Jun N-terminal kinase's role. *J. Mol. Cell Biol.* 5, 277–279. doi: 10.1093/jmcb/mjt015

Steffan, J. S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L. C., Slepko, N., et al. (2004). SUMO modification of huntingtin and huntington's disease pathology. *Science* 304, 100–104. doi: 10.1126/science.1092194

Tiraboschi, P., Hansen, L. A., Thal, L. J., and Corey-Bloom, J. (2004). The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology* 62, 1984–1989. doi: 10.1212/01.WNL.0000129697.01779.0A

Tosun, D., Schuff, N., Mathis, C. A., Jagust, W., and Weiner, M. W. (2011). Spatial patterns of brain amyloid-beta burden and atrophy rate associations in mild cognitive impairment. *Brain* 134, 1077–1088. doi: 10.1093/brain/awr044

Yun, S.-M., Cho, S.-J., Song, J. C., Song, S. Y., Jo, S. A., Jo, C., et al. (2013). SUMO1 modulates Aβ generation via BACE1 accumulation. *Neurobiol. Aging* 34, 650–662. doi: 10.1016/j.neurobiolaging.2012.08.005

Zhang, Y.-Q., and Sarge, K. D. (2008). Sumoylation of amyloid precursor protein negatively regulates Abeta aggregate levels. *Biochem. Biophys. Res. Commun.* 374, 673–678. doi: 10.1016/j.bbrc.2008.07.109

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HSV-1 and Alzheimer's disease: more than a hypothesis

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Among the multiple factors concurring to Alzheimer's disease (AD) pathogenesis, greater attention should be devoted to the role played by infectious agents. Growing epidemiological and experimental evidence suggests that recurrent herpes simplex virus type-1 (HSV-1) infection is a risk factor for AD although the underlying molecular and functional mechanisms have not been fully elucidated yet. Here, we review literature suggesting the involvement of HSV-1 infection in AD also briefly mentioning possible pharmacological implications of these findings.

Keywords: HSV-1, Alzheimer's disease, recurrent infection, γ secretase, amyloid-β protein

ALZHEIMER'S DISEASE, APP PROCESSING AND AMYLOID-β PRODUCTION/ACCUMULATION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline in cognitive functions leading to memory loss and dementia. It involves degeneration of limbic and cortical brain structures, especially in the temporal lobe. In 2010, it was estimated that 35.5 million individuals were affected by dementia in the world with a prediction that this number will increase to 65.7 million by 2030, and 115.4 million by 2050. The World Alzheimer Report 2010 (AD International) estimated that the total costs of dementia in 2010 were US$ 604 billions. Most (>90%) AD cases are sporadic. Only a minority of them have a genetic inheritance and are referred to as familial AD. The main risk factor for AD is aging. The disease afflicts 10% of the population over the age of 65 and 50% of the population over the age of 85. Several factors may increase the chances to develop AD (Imtiaz et al., 2014). Among the "genetic" risk factors, the carriage of a type 4 allele of the apolipoprotein E gene (APOE-ε4) has been widely recognized (see references in Kim et al., 2009). Among the "environmental" risk factors, persistent brain infections, particularly those induced by herpes simplex virus type-1 (HSV-1), seem to play a key role in AD pathogenesis. In a multifactorial disease like AD, to identify the contribution made by each factor and the underlying mechanisms is critical to the development of new therapeutic strategies to prevent the disease and/or slow down its progression.

From the neuropathological point of view, the brains of AD sufferers are characterized by the presence of two major hallmarks mainly located in the hippocampus and cortex: extracellular amyloid plaques composed of insoluble aggregates of the amyloid-β protein (Aβ) and intraneuronal neurofibrillary tangles, composed of hyperphosphorylated Tau protein (Kosik et al., 1986). Numerous other conditions, among which oxidative stress (Mattson et al., 1993) and inflammation, due to microglia activation and astrocytosis, may concur to produce the structural and functional alterations typically found in AD brain (Kitazawa et al., 2004).

Amyloid-β protein is a small protein (generally composed of 40 to 43 amino acids) generated by the proteolytic cleavage of the amyloid precursor protein (APP), a ubiquitous transmembrane protein whose functional role in cell biology has been only partially discovered. APP seems to be involved in neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion and Ca^{2+} signaling (Zheng and Koo, 2006; Octave et al., 2013). Among the various isoforms of APP, due to alternative splicing of *APP* gene located on chromosome 21 in humans, those of 695-amino acid length is prevalent in neurons whereas the other two forms, APP751 and APP770, are expressed in other tissues (Zhang et al., 2011).

Amyloid precursor protein is normally subjected to processing by specific enzymes called "secretases" (Nunan and Small, 2000). It may be processed along two different pathways. The first one, that is most frequent and "non amyloidogenic", involves sequential proteolytic cleavages by the α- and γ-secretases. The former cuts APP at a position 83 amino acids from the C-terminus, thus producing a large N-terminal domain (sAPPα), normally secreted into the extracellular medium, whose function is not well defined. On the opposite site, the other 83-amino-acid C-terminal fragment (C83) is retained in the membrane and it is subsequently cleaved by the γ-secretase complex (that consists of presenilins, nicastrin, anterior pharynx-defective 1 and presenilin enhancer 2), producing a

short fragment that is generally considered non-toxic and known as "p3". The amyloidogenic pathway begins when APP undergoes cleavage by the β-secretase, also known as β-site APP cleaving enzyme 1 or BACE-1 (Vassar et al., 1999). β-secretase cuts APP 16 amino acids before α-secretase and yields two species, the large N-terminal ectodomain of the precursor and the 99-amino acid C-terminus stub (C99). Subsequent cleavage of the latter fragment by γ-secretase results in the formation of Aβ species containing 40 to 42 amino acids. This also means that APP cleavage by α-secretase prevents the Aβ formation. On the other C-terminal side of APP, γ-secretase (in both amyloidogenic and non-amyloidogenic pathways) also generate the "APP intracellular domain" (AICD), that has been reported to modulate the transcription of several genes (including APP itself, BACE-1 and the Aβ-degrading enzyme neprilysin), to regulate apoptosis and contribute to AD pathogenesis (Octave et al., 2013). In APP, the amino acid threonine in position 668 (Thr668) is an important site for its processing. Indeed, APP phosphorylation at Thr668 resulted in increased Aβ formation (Lee et al., 2003; Pierrot et al., 2006). This amino acid is on the C-terminal region of APP and its phosphorylation also regulates the activity of AICD. In fact, when AICD is phosphorylated at Thr668 it interacts with the Fe65 adapter protein (Bórquez and González-Billault, 2012) and enters the nucleus, where it may regulate gene transcription and induce neurodegeneration (Chang et al., 2006).

Amyloidogenic cleavage of APP is not confined to cell membrane; it also takes place in several cellular compartments (Vetrivel and Thinakaran, 2006), including the intermediate compartment of the endoplasmic reticulum (Cook et al., 1997), the trans-Golgi network (Choy et al., 2012), and the endosomal/lysosomal system, where APP processing is regulated by cytoplasmic phosphorylation at Thr668 (Lee et al., 2003). For these reasons, Aβ species may be secreted directly into the extracellular space, where their subsequent aggregation promotes senile plaque formation. Alternatively, it may remain within the cell or may be re-internalized in neurons, where it accumulates (Mohamed and Posse de Chaves, 2011). Some recent findings suggest that Aβ accumulating in neurons, particularly in the form of small oligomers (especially dimers and trimers) is the major determinant of the synaptic damage that highly correlates with the cognitive deficits characterizing the early phases of the disease preceding neuronal death (Tampellini et al., 2007; Mucke and Selkoe, 2012; Ripoli et al., 2013). However, Aβ is not a mere toxic peptide. It is constitutively produced and secreted by cells and, especially when present at very low concentration (in the range of pM), it even supports synapses by increasing synaptic strength in the hippocampus (Puzzo et al., 2008, 2011).

In addition to secretases, APP may be processed by caspases (in particular caspase-3), especially in cells undergoing apoptosis (Gervais et al., 1999; Pellegrini et al., 1999; Fiorelli et al., 2013). In neurons, caspase-induced processing of APP generates a C-terminal fragment (C31) with neurotoxic potential (Lu et al., 2003; Nguyen et al., 2008). Moreover, Fiorelli et al. (2013) showed that caspases cleavage generates two APP fragments (APP-Fs) of 25–35 kDa that are recognized by anti-Aβ antibodies.

Among the genetic risk factors for AD, the carriage of *APOE-ε4* allele plays a major role. Apolipoproteins carry lipids in the circulation and regulate lipid metabolism. ApoE, expressed predominantly in astrocytes, is suggested to be involved in redistribution of cholesterol and phospholipids during membrane remodeling (Holtzman et al., 2012). Links between AD and *APOE-ε4* are multiple: ApoE protein exists in 4 isoforms (E1 to E4), with ApoE3 being the most common allele, and it seems to play a role in Aβ fibrillogenesis and oligomerization as well as in Aβ clearance. Differently from the other three variants, ApoE4 exhibits scarce ability to bind Aβ, thus its expression contributes to Aβ accumulation and aggregation inside neurons and influences the formation of the parenchymal amyloid plaques (Holtzman et al., 2012; Verghese et al., 2013). When human ApoE isoforms were expressed in APP transgenic mice, differences in extracellular Aβ accumulation were observed in an isoform-dependent manner (E2 < E3 < E4; Holtzman et al., 2000). Similar results were also recently obtained by Hashimoto et al. (2012) who reported that the levels of Aβ oligomers in APOE ε4/ε4 AD brains were 2.7 times higher than those found in APOE ε3/ε3 AD brains, and that ApoE increased Aβ oligomer levels in an isoform-dependent manner (E2 < E3 < E4).

Finally, alterations of intracellular Ca^{2+} homeostasis and signaling have also been implicated in AD pathogenesis. It is well known that intracellular Ca^{2+}, besides playing a pivotal role in a large number neuronal functions, is a critical determinant of neuronal survival and death (Piacentini et al., 2008a,b; Maiti et al., 2011; Bading, 2013). Several reports demonstrated correlation between Ca^{2+} homeostasis dysregulation leading to increased intracellular Ca^{2+} levels and AD (Green, 2009). Interestingly, ApoE expression in neuronal and glial cells correlates with increased intracellular Ca^{2+} concentrations in an isoform-dependent manner (E2 < E3 < E4; Müller et al., 1998).

HOW CAN HSV-1 BE INVOLVED IN AD?

Herpes simplex type 1 virus is a neurotropic double-stranded DNA virus that primarily infects epithelial cells of oral and nasal mucosa. Here virus undergoes lytic replication; the newly produced viral particles may enter sensory neurons and, by axonal transport, reach the trigeminal ganglion where usually establishes a latent infection. The virus undergoes periodic reactivation cycles in which the newly formed viral particles are transported back to the site of primary infection through the sensory neurons, causing the well-known clinical lesions (i.e., cold sores and blisters). However, the bipolar trigeminal ganglion neurons also project to the trigeminal nuclei located in the brainstem. From here, neurons project to the thalamus to finally reach the sensory cortex. This is the path through which the reactivated virus may reach the central nervous system (CNS), where it may cause acute neurological disorders like encephalitis [herpes simplex encephalitis (HSE)] or a mild, clinically asymptomatic, infection, or establish life-long latent infection (Kastrukoff et al., 1982; Lewandowski et al., 2002; references in Dobson et al., 2003). The weakening of immune system occurring during aging may favor this process. In addition to the neuronal route, HSV-1 may enter the CNS through the blood stream, as demonstrated by Burgos et al. (2002, 2003, 2005). Many experimental evidence, described below, suggest that accumulation of intracellular damage caused by repeated cycles of viral reactivation may concur to neurodegeneration.

Some reports suggest that during infection herpes virus interacts with several human proteins that it uses to enter the cell and to move from plasma membrane to the nucleus and back (reviewed in Carter, 2008). HSV-1 also uses the host's transcriptional machinery to replicate and binds to proteins that control immune surveillance or apoptosis. Noteworthy, in the attempt to eliminate the virus, host may even cause cell damage via immune and inflammatory responses targeting the virus-containing cells. If this happens in the CNS, HSV-1-induced inflammatory response may result in HSE or, in milder cases, in cell death and neurodegeneration.

Several epidemiological, immunological and molecular evidence link HSV-1 infections to AD pathogenesis. HSV-1 is a ubiquitous virus that affects more than 80% of people over 65 worldwide. The first evidence suggesting the involvement of HSV-1 in AD dates back to 1982 and is based on the observation that people surviving to HSE showed clinical signs reminiscent of AD (i.e., memory loss and cognitive impairment), and that brain regions primarily affected in HSE (limbic system, frontal and temporal cortices) were the same regions compromised in AD (Ball, 1982). During the last 30 years several research groups have conducted many studies providing solid support to the involvement of HSV-1 infection in AD pathogenesis. Here we will briefly summarize the main results of these researches.

EPIDEMIOLOGICAL DATA

Several studies have been performed to assess the presence of HSV-1 in the brain of AD patients. After the first observations made by Ball (1982), other studies have demonstrated that HSE affected the same brain areas involved in AD in humans as well as in rodent experimental models (Damasio and Van Hoesen, 1985; Caparros-Lefebvre et al., 1996; Beffert et al., 1998; Wu et al., 2003; Taylor et al., 2007; Ando et al., 2008). Jamieson et al. (1992) and Itzhaki et al. (1997) reported that a high proportion (about 60%) of brains of elderly people contained latent HSV-1 DNA, especially in the CNS regions critically involved in AD. When present in AD brains, HSV-1 DNA was primarily located within amyloid plaques (Wozniak et al., 2009a). Whether or not the virus contributes to activation of pro-neurodegenerative pathways may largely depend on several host factors, including genetic predisposition, as described in greater detail below. Many infectious agents whose pathogenetic role has been established in other CNS diseases (e.g., varicella zoster virus, causing meningitis or encephalitis; Epstein–Barr virus, associated to both multiple sclerosis and CNS lymphoma; human herpes virus 6, associated to seizure in children) may infect subjects without producing evident clinical signs. On the other hand, even in the presence of "clinically silent" infection, virus may periodically reactivate and replicate in the CNS (Peter and Sevall, 2001; Bearer, 2012). There is no clear evidence on whether HSV-1 reaching the brain and infecting neurons resides there in latent form. However, the detection of HSV-1 DNA in the cerebro-spinal fluid (Deatly et al., 1988; Peter and Sevall, 2001; Plentz et al., 2008) suggests that HSV-1 replicates in the CNS (Bearer, 2012). The presence of HSV-1 proteins in hippocampal neurons of mice infected intraperitoneally with HSV-1 was demonstrated by Burgos et al. (2006a) who also showed that virus is reactivated by hyperthermia. After every reactivation, the newly produced viral particles generated by this "silent" replication and potentially reaching the brain might act, in a drop by drop fashion, to produce local brain damages that may largely differ from those of acute diseases like HSE. It has been recently reported that in brain of infected but asymptomatic mice, HSV-1 reactivation was associated to neuroinflammation and to the appearance of several markers of neurodegeneration including Tau hyperphosphorylation (Martin et al., 2013).

IMMUNOLOGICAL DATA

A number of studies have been conducted to demonstrate the association between AD and HSV-1 infection by searching for antibodies against HSV-1 in the blood of AD patients. These studies revealed a strong correlation between AD occurrence and HSV infection or reactivation, as addressed in a longitudinal study including 512 elderly persons looking for correlation between anti-HSV-1 IgM positivity (a marker of virus primary infection or its reactivation) and development of AD-like cognitive dysfunctions (Letenneur et al., 2008). In the same study, no correlation was found between anti-HSV-1 IgG positivity and early dementia, thus suggesting that recurrent infection, rather the primary one, is dangerous for the CNS. Anti-HSV IgM levels, and not those of IgG, have also been found to inversely correlate with lower plasma Aβ levels (Féart et al., 2011). It is believed that increased amyloid deposition in the brain is paralleled by a lowering of Aβ levels in plasma and that low plasma Aβ levels might be considered possible short-term risk marker of dementia (Schupf et al., 2008; Lambert et al., 2009; Blennow et al., 2010). Another study recently demonstrated that HSV-1 reactivation, assessed as anti-HSV IgG avidity index (that is a more accurate way to demonstrate viral reactivation) occurs in prodromal AD and highly correlates with symptoms of mild cognitive impairment (Kobayashi et al., 2013). Finally, Mancuso et al. (2014) recently found that elevated HSV-1 antibody titers were significantly more frequent in AD patients than in control healthy patients and that they positively correlated with cortical bilateral temporal and orbitofrontal gray matter volume, that may be considered an index of AD pathology (Whitwell et al., 2012).

GENETIC DETERMINANTS

Some studies have suggested that in people carrying the *APOE-ε4* allele and, therefore, predisposed to develop AD, HSV-1 infection markedly increases the risk of AD (Itzhaki et al., 1997; Itzhaki and Lin, 1998; references in Bearer, 2012). However, this correlation was not always confirmed (Beffert et al., 1998). APOE seems to affects the outcome of several different infections (Itzhaki et al., 1998; Dobson et al., 2003) and, interestingly, APOE-ε4 is a risk factor for cold sores (Itzhaki and Wozniak, 2008). Some studies also demonstrated that ApoE4 presence influences the viral load in the brain. Indeed, viral spreading into the brain of ApoE KO mice was lower than that occurring in wild-type mice and correlation was reported between ApoE expression and HSV-1 DNA concentration detected in the CNS (Burgos et al., 2002). In a subsequent study, the same authors showed that during acute infection

with HSV-1, ApoE4 was more efficient than ApoE3 in promoting viral colonization of the brain (Burgos et al., 2006b). However, many genes and proteins implicated in AD, other than ApoE, have been found to interact with herpes simplex viral genome or regulate its life cycle, further supporting the hypothesized synergy between host and pathogens in causing AD-like brain damage (Carter, 2008). From this point of view, genome wide association investigations (GWAs) carried out in a large cohort of AD and non-AD subjects also suggested an infective etiology for sporadic AD. In particular, the polymorphism association of genes located on the chromosome 19 (i.e., Nectin-2 [NC-2]; APOE, glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 [CEACAM-16]; B-cell lymphoma-3 [Bcl-3]; translocase of outer mitochondrial membrane 40 homolog [TOMM-40]) and on the chromosome 8 (complement receptor-1 [CR-l]; APOJ, C-type lectin domain A family-16 member [CLEC-16A]), results in a genetic fingerprint that might modulate brain susceptibility to herpes virus infection and lead to neuronal loss, inflammation and Aβ deposition (Porcellini et al., 2010; Licastro et al., 2011).

MOLECULAR DATA

Several studies demonstrated that HSV-1 affects APP processing. HSV-1 infection may have profound effects on the host intracellular pathways leading to activation/inactivation of several signaling molecules and kinases involved in APP metabolism. Ruth Itzhaki's group found that in human SH-SY5Y neuroblastoma cells HSV-1 induces APP cleavage with the production of a 55-kDa fragment (recognized in Western blot analysis by an anti-C-terminal APP antibody) starting from 6 h post infection (hpi), and the concomitant reduction of band intensity relative to full-length APP (Shipley et al., 2005). These authors also reported that HSV-1 infection caused Aβ40 and Aβ42 accumulation in human neuroblastoma and glioblastoma cells *in vitro*, whereas in brains of mice infected with HSV-1 they only found an increase in Aβ42 (Wozniak et al., 2007). However, further studies on *in vivo* models of recurrent HSV-1 brain infections are needed to determine the structural and functional alterations induced by viral reactivation.

We have demonstrated that in cultured neuronal cells HSV-1 induces amyloidogenic APP cleavage, with production of several APP-Fs including Aβ (De Chiara et al., 2010; Piacentini et al., 2011). In particular, we found that infection of SH-SY5Y human neuroblastoma cells and rat cortical neurons with HSV-1 induces multiple cleavages of APP, which result in the intra- and/or extracellular accumulation of several APP-Fs with neurotoxic potential. Among them we found: (i) APP-Fs of 35 and 45 kDa (APP-F35 and APP-F45) that comprise portions of Aβ; (ii) N-terminal APP-Fs that are secreted extracellularly; (iii) intracellular C-terminal APP-Fs, including AICD; and (iv) Aβ40 and Aβ42 in the form of monomers and small oligomers (dimers and trimers). Notably, the fragment APP-F35 seems to be a large Aβ oligomer (probably a nonamer) that is typical of viral-induced APP processing given that it was only found in cells infected with HSV-1. Indeed, it was revealed with antibodies recognizing amino-acidic sequences in Aβ (e.g., 4G8 and M2°, targeting amino acids 17–24 and 1–10 of Aβ, respectively), only 8–18 hpi (**Figures 1** and **2A**). Moreover, its formation was inhibited in the presence of β- and γ-secretase inhibitors. APP-F45 was revealed by M2° only. On the contrary, neither of these APP-Fs were recognized by antibodies raised against the N- and C-terminals of APP (**Figure 1**), thus indicating that they are not APP cleavage end-products. We also demonstrated that APP-Fs were present in the extracellular space and they had the ability to induce apoptosis in the neighboring cells (De Chiara et al., 2010). The multiple cleavages of APP occurring in infected cells are produced in part by known components of the amyloidogenic APP processing pathway, i.e., host-cell β-secretase, γ-secretase, and caspase-3-like enzymes, and in part by other cellular or viral enzymes not yet identified. Incidentally, the increased production of Aβ also reflected in an increased production and nuclear accumulation of AICD fragment that it is known to play a role in AD by modulating gene transcription.

It has been hypothesized that viral glycoprotein B is responsible for Aβ aggregation because this glycoprotein shares a significant portion of amino acid sequence similarity with Aβ, and it might serve as "core" to trigger Aβ aggregation (Cribbs et al., 2000). However, we and others (Wozniak et al., 2007; Piacentini et al., 2011) found that HSV-1-induced increased Aβ immunoreactivity was not due to cross-reactivity of the anti-Aβ antibodies with this glycoprotein.

From a functional point of view, we reported that HSV-1 binding to neuronal membrane induced membrane depolarization leading to increased neuronal excitability and triggering action potentials. This depolarization was due to activation of persistent Na^+ currents and inhibition of leak K^+ currents. Neuronal hyperexcitability persisted over time and was observed in neurons also at 12 hpi. Downstream to this effect we observed increased intracellular Ca^{2+} signaling, mainly due to activation of L-type Ca^{2+} channels and opening of inositol trisphosphate receptors ($InsP_3Rs$) that caused marked intracellular Ca^{2+} entry from extracellular medium and Ca^{2+} release from intracellular stores (Piacentini et al., 2011). As above described, this Ca^{2+} dysregulation may trigger neurodegeneration (Mattson and Chan, 2003; Piacentini et al., 2008a,b; Chakroborty et al., 2012). We also found that HSV-1 induced Ca^{2+}-mediated APP phosphorylation at Thr668. This is a key event critically involved in APP processing and Aβ formation (Pierrot et al., 2006). We demonstrated that HSV-1 infection induces a significant accumulation of Aβ42 inside neurons (**Figure 2**), and this effect depended on Ca^{2+} signaling activation. Indeed, in the presence of nifedipine and/or 2-aminoethoxydiphenyl borate (2-APB), that are specific blockers of L-type Ca^{2+} channels and $InsP_3$ receptors, respectively, intraneuronal Aβ accumulation was significantly reduced (Piacentini et al., 2011). Besides accumulating inside neurons, Aβ monomers and small oligomers (dimers and trimers) were also released in the culture medium of infected neurons and revealed by Western blot analysis.

Several other studies have investigated the link between HSV-1 and AD at molecular level, independently on APP processing. It has been demonstrated that HSV-1 infection causes several functional and molecular alterations including: neurodegeneration and AD-like phosphorylation of tau protein (Zambrano et al., 2008; Wozniak et al., 2009b), the latter involving the activation of the glicogen sinthase kinase 3; caspase-3-mediated cleavage of Tau

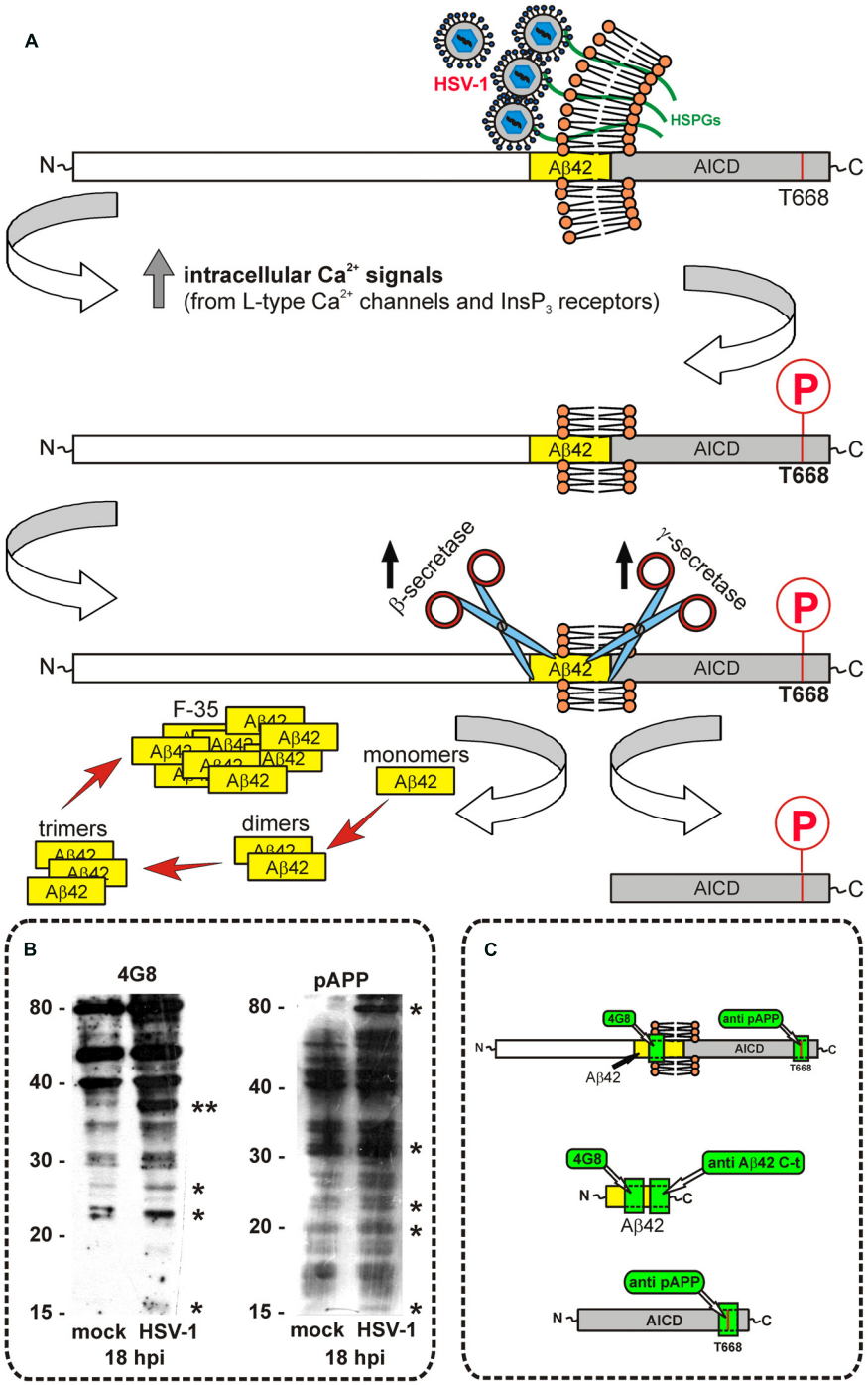

FIGURE 1 | HSV-1 infection induces APP cleavage. (A) Our data suggest that HSV-1 binding to the heparan sulfate proteoaminoglycans (HSPGs) expressed on neuronal plasma membrane induces Ca^{2+} signals triggering APP phosphorylation at Thr668 that, in turn, increases β- and γ-secretase activity. As a result, there is an increased production of Aβ that aggregates to form oligomers of various size. The C-terminal APP fragment called AICD, created by the cleavage of γ secretase is phosphorylated at Thr668 and internalized in the nucleus where it may modulate gene transcription. **(B)** Western blots showing representative experiments performed with 4G8 antibody and anti $pAPP^{Thr668}$ on intracellular lysates of neuronal cells. Asterisks in the blots indicate bands that are modified by 18 h of HSV-1 infection (hours post infection, hpi) with respect to the mock-infected conditions. Double asterisk in the Western blot for 4G8 indicates the APP fragment F35, characteristic of HSV-1 infected cells. **(C)** Cartoon indicating the amino acid sequences targeted by the different antibodies we used for Western blot and immunofluorescence experiments: 4G8 recognizes all APP fragments containing the amino acid sequence 17–24 of Aβ, independently on its cleavage. Therefore, it reacts with APP "full-length", and with all APP fragments that contain Aβ 17–24 sequence; $pAPP^{Thr668}$ antibody reacts with all C-terminus APP fragments containing phosphorylated Thr668, including APP full-length; anti Aβ42 C-terminus specifically reacts with the C-terminal part of Aβ42, and it does not recognize APP. Western blots in the panel **B** refer to previously published data (De Chiara et al., 2010).

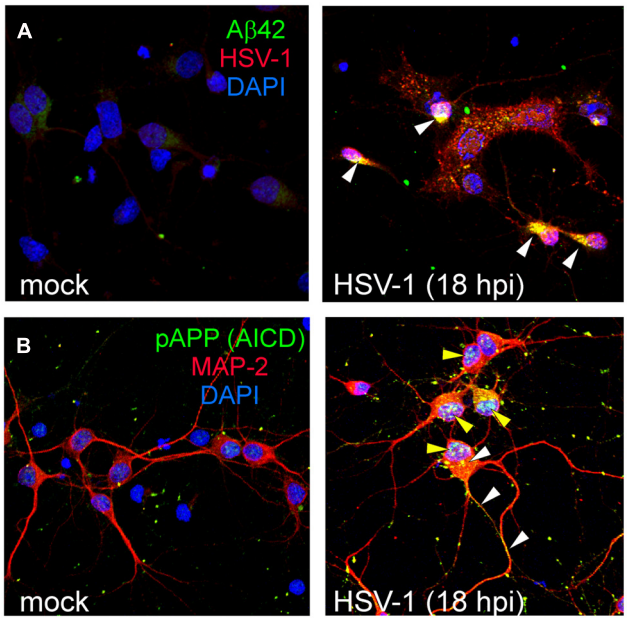

FIGURE 2 | HSV-1 infection induces intracellular accumulation of several APP fragments. Representative examples of mock and HSV-1-infected (18 hpi) rat cortical neurons immunostained for Aβ42 C-terminus (green) and HSV-1 (red; **A)**; $pAPP^{Thr668}$/$AICD^{pThr668}$ (green) and MAP-2 (red; **B)**. Nuclei are stained in blue with DAPI. White arrowheads in the panel **A** (right) indicate intracellular accumulation of Aβ42 in infected (HSV-1-positive) neurons. In the panel **B** (right), yellow arrowheads indicate nuclear accumulation of $AICD^{pThr668}$ in infected neurons (MAP-2-positive) whereas white arrowheads indicate staining for putative APP full-length phosphorylated at Thr668. Images in the panels **A** and **B** refer to previously published data (Piacentini et al., 2011).

(Lerchundi et al., 2011); activation of the arachidonic acid cascade, which is involved in AD-type neuropathological changes (Hill et al., 2009). With regard to the interaction between herpes simplex viruses and APP, it has been recently shown that HSV-1 alters the distribution of cellular APP, and APP and HSV-1 capsid proteins physically interact to allow the migration of new viral particles inside infected cells by fast anterograde transport mechanisms (Cheng et al., 2011). This also suggests that HSV-1 affects APP dynamic and its intracellular distribution thus possibly causing alterations in its metabolism and processing.

POSSIBLE HSV-1 RELATED THERAPEUTIC INTERVENTIONS

Experimental evidence reviewed above allows us to hypothesize that, in the near future, treatments aimed at preventing and/or delaying AD might include antiviral agents and/or target HSV-1-activated intracellular pathways. At the present, the main antiviral agent used for HSV-1 is Acyclovir. Acyclovir, that is the common name of acycloguanosine, targets infected cells and inhibits viral replication (Park et al., 1979). Wozniak et al. (2011) demonstrated that acyclovir reduced Aβ formation and Tau phosphorylation *in vitro*. This finding suggests that the appearance of molecular AD hallmarks depends on HSV-1 replication. Similarly, we observed that in rat cortical neurons, UV-inactivation of HSV-1 particles prevented Aβ42 formation and its intracellular accumulation, although the virus retained the ability to bind the neuronal membrane and trigger intracellular signaling leading to short-term APP phosphorylation (Piacentini et al., 2011). Itzhaki suggested that treatment with a variant of acyclovir, the valacyclovir, could be of great interest in treatment of HSV-1-induced neurodegeneration (Itzhaki and Wozniak, 2012). Indeed, valacyclovir displays greater bioavailability than acyclovir (5-fold to 10-fold), it crosses the blood brain barrier and has no toxicity when used in patients with multiple sclerosis (Friedman et al., 2005). Interestingly, lysine supplementation has been suggested to have a role in preventing the development of AD by reducing HSV-1 replication (Rubey, 2010).

Among the proposed pharmacological treatments for AD, the use of statins seems to be promising (Barone et al., 2013). It has been suggested that their beneficial effects may be related to their ability to modulate the entry of pathogens given that cholesterol synthesis inhibition blocks the entry, and limits neuronal spread, of HSV-1 (Hill et al., 2005).

Finally, some studies also suggested the use of GSK-3β inhibitors as possible candidates for AD treatment (King et al., 2014). Among them, lithium has been shown to have some beneficial effects on AD symptoms (see references in Forlenza et al., 2012; Nunes et al., 2013). Lithium has also been reported to inhibit HSV-1 replication both *in vitro* and *in vivo* (Ziaie and Kefalides, 1989; Ziaie et al., 1994; Amsterdam et al., 1996), thus allowing to speculate that its beneficial effects might also depend on its antiviral activity.

CONCLUSION

Data reviewed here support the hypothesis that recurrent HSV-1 infection in the brain may have a critical role in AD pathogenesis by directly activating intracellular pathways leading to typical AD molecular hallmarks. Studies ongoing in our laboratory on *in vivo* models of recurrent mild brain HSV-1 infection will expectedly further support the causal relationship between HSV-1 reactivation in the CNS and AD-like cognitive decline. Collectively, data discussed in this manuscript indicate that greater attention should be paid to infectious and, especially, viral agents among the environmental factors contributing to AD pathogenesis.

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REFERENCES

Amsterdam, J. D., Maislin, G., and Hooper, M. B. (1996). Suppression of herpes simplex virus infections with oral lithium carbonate – a possible antiviral activity. *Pharmacotherapy* 16, 1070–1075.

Ando, Y., Kitayama, H., Kawaguchi, Y., and Koyanagi, Y. (2008). Primary target cells of herpes simplex virus type 1 in the hippocampus. *Microbes Infect.* 10, 1514–1523. doi: 10.1016/j.micinf.2008.09.005

Bading, H. (2013). Nuclear calcium signalling in the regulation of brain function. *Nat. Rev. Neurosci.* 14, 593–608. doi: 10.1038/nrn3531

Ball, M. J. (1982). Limbic predilection in Alzheimer dementia: is reactivated herpesvirus involved? *Can. J. Neurol. Sci.* 9, 303–306.

Barone, E., Di Domenico, F., and Butterfield, D. A. (2013). Statins more than cholesterol lowering agents in Alzheimer disease: their pleiotropic functions as potential therapeutic targets. *Biochem. Pharmacol.* 88, 605–616. doi: 10.1016/j.bcp.2013.10.030

Bearer, E. L. (2012). HSV, axonal transport and Alzheimer's disease: *in vitro* and *in vivo* evidence for causal relationships. *Future Virol.* 7, 885–899. doi: 10.2217/fvl.12.81

Beffert, U., Bertrand, P., Champagne, D., Gauthier, S., and Poirier, J. (1998). HSV-1 in brain and risk of Alzheimer's disease. *Lancet* 351, 1330–1331. doi: 10.1016/S0140-6736(05)79057-7

Blennow, K., Hampel, H., Weiner, M., and Zetterberg, H. (2010). Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat. Rev. Neurol.* 6, 131–144. doi: 10.1038/nrneurol.2010.4

Bórquez, D. A., and González-Billault, C. (2012). The amyloid precursor protein intracellular domain-fe65 multiprotein complexes: a challenge to the amyloid hypothesis for Alzheimer's disease? *Int. J. Alzheimers Dis.* 2012, 353145. doi: 10.1155/2012/353145

Burgos, J. S., Ramirez, C., Sastre, I., Bullido, M. J., and Valdivieso, F. (2002). Involvement of apolipoprotein E in the hematogenous route of herpes simplex virus type 1 to the central nervous system. *J. Virol.* 76, 12394–12398. doi: 10.1128/JVI.76.23.12394-12398.2002

Burgos, J. S., Ramirez, C., Sastre, I., Bullido, M. J., and Valdivieso, F. (2003). ApoE4 is more efficient than E3 in brain access by herpes simplex virus type 1. *Neuroreport* 14, 1825–1827. doi: 10.1097/00001756-200310060-00013

Burgos, J. S., Ramirez, C., Sastre, I., Alfaro, J. M., and Valdivieso, F. (2005). Herpes simplex virus type 1 infection via the bloodstream with apolipoprotein E dependence in the gonads is influenced by gender. *J. Virol.* 79, 1605–1612. doi: 10.1128/JVI.79.3.1605-1612.2005

Burgos, J. S., Ramirez, C., Guzman-Sanchez, F., Alfaro, J. M., Sastre, I., and Valdivieso, F. (2006a). Hematogenous vertical transmission of herpes simplex virus type 1 in mice. *J. Virol.* 80, 2823–2831. doi: 10.1128/JVI.80.6.2823-2831.2006

Burgos, J. S., Ramirez, C., Sastre, I., and Valdivieso, F. (2006b). Effect of apolipoprotein E on the cerebral load of latent herpes simplex virus type 1 DNA. *J. Virol.* 80, 5383–5387. doi: 10.1128/JVI.00006-06

Caparros-Lefebvre, D., Girard-Buttaz, I., Reboul, S., Lebert, F., Cabaret, M., Verier, A., et al. (1996). Cognitive and psychiatric impairment in herpes simplex virus encephalitis suggest involvement of the amygdalo-frontal pathways. *J. Neurol.* 243, 248–256. doi: 10.1007/BF00868522

Carter, C. J. (2008). Interactions between the products of the Herpes simplex genome and Alzheimer's disease susceptibility genes: relevance to pathological-signalling cascades. *Neurochem. Int.* 52, 920–934. doi: 10.1016/j.neuint.2007.11.003

Chakroborty, S., Briggs, C., Miller, M. B., Goussakov, I., Schneider, C., Kim, J., et al. (2012). Stabilizing ER Ca^{2+} channel function as an early preventative strategy for Alzheimer's disease. *PLoS ONE* 7:e52056. doi: 10.1371/journal.pone.0052056

Chang, K. A., Kim, H. S., Ha, T. Y., Ha, J. W., Shin, K. Y., Jeong, Y. H., et al. (2006). Phosphorylation of amyloid precursor protein (APP) at Thr668 regulates the nuclear translocation of the APP intracellular domain and induces neurodegeneration. *Mol. Cell. Biol.* 26, 4327–4338. doi: 10.1128/MCB.02393-05

Cheng, S. B., Ferland, P., Webster, P., and Bearer, E. L. (2011). Herpes simplex virus dances with amyloid precursor protein while exiting the cell. *PLoS ONE* 6:e17966. doi: 10.1371/journal.pone.0017966

Choy, R. W., Cheng, Z., and Schekman, R. (2012). Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2077–E2082. doi: 10.1073/pnas.1208635109

Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., et al. (1997). Alzheimer's Aβ(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat. Med.* 3, 1021–1023.

Cribbs, D. H., Azizeh, B. Y., Cotman, C. W., and LaFerla, F. M. (2000). Fibril formation and neurotoxicity by a herpes simplex virus glycoprotein B fragment with homology to the Alzheimer's Aβ peptide. *Biochemistry* 39, 5988–5994.

Damasio, A. R., and Van Hoesen, G. W. (1985). The limbic system and the localisation of herpes simplex encephalitis. *J. Neurol. Neurosurg. Psychiatry* 48, 297–301. doi: 10.1136/jnnp.48.4.297

Deatly, A. M., Spivack, J. G., Lavi, E., O'Boyle, D. R. II, and Fraser, N. W. (1988). Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. *J. Virol.* 62, 749–756.

De Chiara, G., Marcocci, M. E., Civitelli, L., Argnani, R., Piacentini, R., Ripoli, C., et al. (2010). APP processing induced by herpes simplex virus type 1 (HSV-1) yields several APP fragments in human and rat neuronal cells. *PLoS ONE* 5:e13989. doi: 10.1371/journal.pone.0013989

Dobson, C. B., Wozniak, M. A., and Itzhaki, R. F. (2003). Do infectious agents play a role in dementia? *Trends Microbiol.* 11, 312–317. doi: 10.1016/S0966-842X(03)00146-X

Féart, C., Helmer, C., Fleury, H., Béjot, Y., Ritchie, K., Amouyel, P., et al. (2011). Association between IgM anti-herpes simplex virus and plasma amyloid-β levels. *PLoS ONE* 6:e29480. doi: 10.1371/journal.pone.0029480

Fiorelli, T., Kirouac, L., and Padmanabhan, J. (2013). Altered processing of amyloid precursor protein in cells undergoing apoptosis. *PLoS ONE* 8:e57979. doi: 10.1371/journal.pone.0057979

Forlenza, O. V., de Paula, V. J., Machado-Vieira, R., Diniz, B. S., and Gattaz, W. F. (2012). Does lithium prevent Alzheimer's disease? *Drugs Aging* 29, 335–342. doi: 10.2165/11599180-000000000-00000

Friedman, J. E., Zabriskie, J. B., Plank, C., Ablashi, D., Whitman, J., Shahan, B., et al. (2005). A randomized clinical trial of valacyclovir in multiple sclerosis. *Mult. Scler.* 11, 286–295. doi: 10.1191/1352458505ms1185oa

Gervais, F. G., Xu, D., Robertson, G. S., Vaillancourt, J. P., Zhu, Y., Huang, J., et al. (1999). Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-β precursor protein and amyloidogenic Aβ peptide formation. *Cell* 97, 395–406. doi: 10.1016/S0092-8674(00)80748-5

Green, K. N. (2009). Calcium in the initiation, progression and as an effector of Alzheimer's disease pathology. *J. Cell. Mol. Med.* 13, 2787–2799. doi: 10.1111/j.1582-4934.2009.00861.x

Hashimoto, T., Serrano-Pozo, A., Hori, Y., Adams, K. W., Takeda, S., Banerji, A. O., et al. (2012). Apolipoprotein E, especially apolipoprotein E4, increases the oligomerization of amyloid β peptide. *J. Neurosci.* 32, 15181–15192. doi: 10.1523/JNEUROSCI.1542-12.2012

Hill, J. M., Steiner, I., Matthews, K. E., Trahan, S. G., Foster, T. P., and Ball, M. J. (2005). Statins lower the risk of developing Alzheimer's disease by limiting lipid raft endocytosis and decreasing the neuronal spread of Herpes simplex virus type 1. *Med. Hypotheses* 64, 53–58. doi: 10.1016/j.mehy.2003.12.058

Hill, J. M., Zhao, Y., Clement, C., Neumann, D. M., and Lukiw, W. J. (2009). HSV-1 infection of human brain cells induces miRNA-146a and Alzheimer-type inflammatory signaling. *Neuroreport* 20, 1500–1505. doi: 10.1097/WNR.0b013e3283329c05

Holtzman, D. M., Bales, K. R., Tenkova, T., Fagan, A. M., Parsadanian, M., Sartorius, L. J., et al. (2000). Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2892–2897. doi: 10.1073/pnas.050004797

Holtzman, D. M., Herz, J., and Bu, G. (2012). Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2, a006312. doi: 10.1101/cshperspect.a006312

Imtiaz, B., Tolppanen, A. M., Kivipelto, M., and Soininen, H. (2014). Future directions in Alzheimer's disease from risk factors to prevention. *Biochem. Pharmacol.* 88, 661–670. doi: 10.1016/j.bcp.2014.01.003

Itzhaki, R. F., and Lin, W. R. (1998). Herpes simplex virus type I in brain and the type 4 allele of the apolipoprotein E gene are a combined risk factor for Alzheimer's disease. *Biochem. Soc. Trans.* 26, 273–277.

Itzhaki, R. F., Lin, W. R., Shang, D., Wilcock, G. K., Faragher, B., and Jamieson, G. A. (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* 349, 241–244. doi: 10.1016/S0140-6736(96)10149-5

Itzhaki, R., and Wozniak, M. (2008). Susceptibility to herpes simplex labialis conferred by the gene encoding apolipoprotein E. *J. Infect. Dis.* 198, 624–625; author reply 625–626. doi: 10.1086/590213

Itzhaki, R. F., Wozniak, M., Dobson, C., and Lin, W. R. (1998). ApoE-viral interactions. *Nat. Med.* 4, 1344. doi: 10.1038/3908

Itzhaki, R. F., and Wozniak, M. A. (2012). Could antivirals be used to treat Alzheimer's disease? *Future Microbiol.* 7, 307–309. doi: 10.2217/fmb.12.10

Jamieson, G. A., Maitland, N. J., Wilcock, G. K., Yates, C. M., and Itzhaki, R. F. (1992). Herpes simplex virus type 1 DNA is present in specific regions of brain from aged people with and without senile dementia of the Alzheimer type. *J. Pathol.* 167, 365–368. doi: 10.1002/path.1711670403

Kastrukoff, L., Hamada, T., Schumacher, U., Long, C., Doherty, P. C., and Koprowski, H. (1982). Central nervous system infection and immune response in mice inoculated into the lip with herpes simplex virus type 1. *J. Neuroimmunol.* 2, 295–305. doi: 10.1016/0165-5728(82)90062-5

Kim, J., Basak, J. M., and Holtzman, D. M. (2009). The role of apolipoprotein E in Alzheimer's disease. *Neuron* 63, 287–303. doi: 10.1016/j.neuron.2009.06.026

King, M. K., Pardo, M., Cheng, Y., Downey, K., Jope, R. S., and Beurel, E. (2014). Glycogen synthase kinase-3 inhibitors: rescuers of cognitive impairments. *Pharmacol. Ther.* 141, 1–12. doi: 10.1016/j.pharmthera.2013.07.010

Kitazawa, M., Yamasaki, T. R., and LaFerla, F. M. (2004). Microglia as a potential bridge between the amyloid β-peptide and tau. *Ann. N. Y. Acad. Sci.* 1035, 85–103. doi: 10.1196/annals.1332.006

Kobayashi, N., Nagata, T., Shinagawa, S., Oka, N., Shimada, K., Shimizu, A., et al. (2013). Increase in the IgG avidity index due to herpes simplex virus type 1 reactivation and its relationship with cognitive function in amnestic mild cognitive impairment and Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 430, 907–911. doi: 10.1016/j.bbrc.2012.12.054

Kosik, K. S., Joachim, C. L., and Selkoe, D. J. (1986). Microtubule-associated protein tau (τ) is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* 83, 4044–4048. doi: 10.1073/pnas.83.11.4044

Lambert, J. C., Schraen-Maschke, S., Richard, F., Fievet, N., Rouaud, O., Berr, C., et al. (2009). Association of plasma amyloid β with risk of dementia: the prospective Three-City Study. *Neurology* 73, 847–853. doi: 10.1212/WNL.0b013e3181b78448

Lee, M. S., Kao, S. C., Lemere, C. A., Xia, W., Tseng, H. C., Zhou, Y., et al. (2003). APP processing is regulated by cytoplasmic phosphorylation. *J. Cell Biol.* 163, 83–95. doi: 10.1083/jcb.200301115

Lerchundi, R., Neira, R., Valdivia, S., Vio, K., Concha, M. I., Zambrano, A., et al. (2011). Tau cleavage at D421 by caspase-3 is induced in neurons and astrocytes infected with herpes simplex virus type 1. *J. Alzheimers Dis.* 23, 513–520. doi: 10.3233/JAD-2010-101386

Letenneur, L., Pérès, K., Fleury, H., Garrigue, I., Barberger-Gateau, P., Helmer, C., et al. (2008). Seropositivity to herpes simplex virus antibodies and risk of Alzheimer's disease: a population-based cohort study. *PLoS ONE* 3:e3637. doi: 10.1371/journal.pone.0003637

Lewandowski, G., Zimmerman, M. N., Denk, L. L., Porter, D. D., and Prince, G. A. (2002). Herpes simplex type 1 infects and establishes latency in the brain and trigeminal ganglia during primary infection of the lip in cotton rats and mice. *Arch. Virol.* 147, 167–179. doi: 10.1007/s705-002-8309-9

Licastro, F., Carbone, I., Ianni, M., and Porcellini, E. (2011). Gene signature in Alzheimer's disease and environmental factors: the virus chronicle. *J. Alzheimers Dis.* 27, 809–817. doi: 10.3233/JAD-2011-110755

Lu, D. C., Soriano, S., Bredesen, D. E., and Koo, E. H. (2003). Caspase cleavage of the amyloid precursor protein modulates amyloid β-protein toxicity. *J. Neurochem.* 87, 733–741. doi: 10.1046/j.1471-4159.2003.02059.x

Maiti, P., Piacentini, R., Ripoli, C., Grassi, C., and Bitan, G. (2011). Surprising toxicity and assembly behaviour of amyloid β-protein oxidized to sulfone. *Biochem. J.* 433, 323–332. doi: 10.1042/BJ20101391

Mancuso, R., Baglio, F., Cabinio, M., Calabrese, E., Hernis, A., Nemni, R., et al. (2014). Titers of herpes simplex virus type 1 antibodies positively correlate with grey matter volumes in Alzheimer's disease. *J. Alzheimers Dis.* 38, 741–745. doi: 10.3233/JAD-130977

Martin, C., Aguila, B., Araya, P., Vio, K., Valdivia, S., Zambrano, A., et al. (2013). Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation. *J. Alzheimers Dis.* 39, 849–859. doi: 10.3233/JAD-131706

Mattson, M. P., Barger, S. W., Cheng, B., Lieberburg, I., Smith-Swintosky, V. L., and Rydel, R. E. (1993). β-Amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. *Trends Neurosci.* 16, 409–414. doi: 10.1016/0166-2236(93)90009-B

Mattson, M. P., and Chan, S. L. (2003). Calcium orchestrates apoptosis. *Nat. Cell Biol.* 5, 1041–1043. doi: 10.1038/ncb1203-1041

Mohamed, A., and Posse de Chaves, E. (2011). Aβ internalization by neurons and glia. *Int. J. Alzheimers Dis.* 2011, 127984. doi: 10.4061/2011/127984

Müller, W., Meske, V., Berlin, K., Scharnagl, H., März, W., and Ohm, T. G. (1998). Apolipoprotein E isoforms increase intracellular Ca^{2+} differentially through a omega-agatoxin IVa-sensitive Ca^{2+}-channel. *Brain Pathol.* 8, 641–653. doi: 10.1111/j.1750-3639.1998.tb00190.x

Mucke, L., and Selkoe, D. J. (2012). Neurotoxicity of amyloid β-protein: synaptic and network dysfunction. *Cold Spring Harb. Perspect Med.* 2, a006338. doi: 10.1101/cshperspect.a006338

Nguyen, T. V., Galvan, V., Huang, W., Banwait, S., Tang, H., Zhang, J., et al. (2008). Signal transduction in Alzheimer disease: p21-activated kinase signaling requires C-terminal cleavage of APP at Asp664. *J. Neurochem.* 104, 1065–1080. doi: 10.1111/j.1471-4159.2007.05031.x

Nunan, J., and Small, D. H. (2000). Regulation of APP cleavage by α-, β- and γ-secretases. *FEBS Lett.* 483, 6–10. doi: 10.1016/S0014-5793(00)02076-7

Nunes, M. A., Viel, T. A., and Buck, H. S. (2013). Microdose lithium treatment stabilized cognitive impairment in patients with Alzheimer's disease. *Curr. Alzheimer Res.* 10, 104–107. doi: 10.2174/156720513804871354

Octave, J. N., Pierrot, N., Ferao Santos, S., Nalivaeva, N. N., and Turner, A. J. (2013). From synaptic spines to nuclear signaling: nuclear and synaptic actions of the amyloid precursor protein. *J. Neurochem.* 126, 183–190. doi: 10.1111/jnc.12239

Park, N. H., Pavan-Langston, D., and McLean, S. L. (1979). Acylovir in oral and ganglionic herpes simplex virus infections. *J. Infect. Dis.* 140, 802–806. doi: 10.1093/infdis/140.5.802

Pellegrini, L., Passer, B. J., Tabaton, M., Ganjei, J. K., and D'Adamio, L. (1999). Alternative, non-secretase processing of Alzheimer's β-amyloid precursor protein during apoptosis by caspase-6 and -8. *J. Biol. Chem.* 274, 21011–21016. doi: 10.1074/jbc.274.30.21011

Peter, J. B., and Sevall, J. S. (2001). Review of 3200 serially received CSF samples submitted for type-specific HSV detection by PCR in the reference laboratory setting. *Mol. Cell. Probes* 15, 177–182. doi: 10.1006/mcpr.2001.0356

Piacentini, R., Civitelli, L., Ripoli, C., Marcocci, M. E., De Chiara, G., Garaci, E., et al. (2011). HSV-1 promotes Ca^{2+} -mediated APP phosphorylation and Aβ accumulation in rat cortical neurons. *Neurobiol. Aging* 32, 2323.e13–2323.e26.

Piacentini, R., Gangitano, C., Ceccariglia, S., Del Fà, A., Azzena, G. B., Michetti, F., et al. (2008a). Dysregulation of intracellular calcium homeostasis is responsible for neuronal death in an experimental model of selective hippocampal degeneration induced by trimethyltin. *J. Neurochem.* 105, 2109–2121. doi: 10.1111/j.1471-4159.2008.05297.x

Piacentini, R., Ripoli, C., Leone, L., Misiti, F., Clementi, M. E., D'Ascenzo, M., et al. (2008b). Role of methionine 35 in the intracellular Ca^{2+} homeostasis dysregulation and Ca^{2+}-dependent apoptosis induced by amyloid β-peptide in human neuroblastoma IMR32 cells. *J. Neurochem.* 107, 1070–1082.

Pierrot, N., Santos, S. F., Feyt, C., Morel, M., Brion, J. P., and Octave, J. N. (2006). Calcium-mediated transient phosphorylation of tau and amyloid precursor protein followed by intraneuronal amyloid-β accumulation. *J. Biol. Chem.* 281, 39907–39914. doi: 10.1074/jbc.M606015200

Plentz, A., Jilg, W., Kochanowski, B., Ibach, B., and Knöll, A. (2008). Detection of herpesvirus DNA in cerebrospinal fluid and correlation with clinical symptoms. *Infection* 36, 158–162. doi: 10.1007/s15010-007-6354-y

Porcellini, E., Carbone, I., Ianni, M., and Licastro, F. (2010). Alzheimer's disease gene signature says: beware of brain viral infections. *Immun. Ageing* 7, 16. doi: 10.1186/1742-4933-7-16

Puzzo, D., Privitera, L., Fa', M., Staniszewski, A., Hashimoto, G., Aziz, F., et al. (2011). Endogenous amyloid-β is necessary for hippocampal synaptic plasticity and memory. *Ann. Neurol.* 69, 819–830. doi: 10.1002/ana.22313

Puzzo, D., Privitera, L., Leznik, E., Fà, M., Staniszewski, A., Palmeri, A., et al. (2008). Picomolar amyloid-β positively modulates synaptic plasticity and memory in hippocampus. *J. Neurosci.* 28, 14537–14545. doi: 10.1523/JNEUROSCI.2692-08.2008

Ripoli, C., Piacentini, R., Riccardi, E., Leone, L., Li Puma, D. D., Bitan, G., et al. (2013). Effects of different amyloid β-protein analogues on synaptic function. *Neurobiol. Aging* 34, 1032–1044. doi: 10.1016/j.neurobiolaging.2012.06.027

Rubey, R. N. (2010). Could lysine supplementation prevent Alzheimer's dementia? A novel hypothesis. *Neuropsychiatr. Dis. Treat.* 6, 707–710. doi: 10.2147/NDT.S14338

Schupf, N., Tang, M. X., Fukuyama, H., Manly, J., Andrews, H., Mehta, P., et al. (2008). Peripheral Aβ subspecies as risk biomarkers of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14052–14057. doi: 10.1073/pnas.0805902105

Shipley, S. J., Parkin, E. T., Itzhaki, R. F., and Dobson, C. B. (2005). Herpes simplex virus interferes with amyloid precursor protein processing. *BMC Microbiol.* 5:48. doi: 10.1186/1471-2180-5-48

Tampellini, D., Magrané, J., Takahashi, R. H., Li, F., Lin, M. T., Almeida, C. G., et al. (2007). Internalized antibodies to the Aβ domain of APP reduce neuronal Aβ and protect against synaptic alterations. *J. Biol. Chem.* 282, 18895–18906. doi: 10.1074/jbc.M700373200

Taylor, S. W., Lee, D. H., and Jackson, A. C. (2007). Herpes simplex encephalitis presenting with exclusively frontal lobe involvement. *J. Neurovirol.* 13, 477–481. doi: 10.1080/13550280701491131

Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., et al. (1999). β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286, 735–741. doi: 10.1126/science.286.5440.735

Verghese, P. B., Castellano, J. M., Garai, K., Wang, Y., Jiang, H., Shah, A., et al. (2013). ApoE influences amyloid-β (Aβ) clearance despite minimal apoE/Aβ association in physiological conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1807–E1816. doi: 10.1073/pnas.1220484110

Vetrivel, K. S., and Thinakaran, G. (2006). Amyloidogenic processing of β-amyloid precursor protein in intracellular compartments. *Neurology* 66, S69–S73. doi: 10.1212/01.wnl.0000192107.17175.39

Whitwell, J. L., Dickson, D. W., Murray, M. E., Weigand, S. D., Tosakulwong, N., Senjem, M. L., et al. (2012). Neuroimaging correlates of pathologically defined subtypes of Alzheimer's disease: a case-control study. *Lancet Neurol.* 11, 868–877. doi: 10.1016/S1474-4422(12)70200-4

Wozniak, M. A., Mee, A. P., and Itzhaki, R. F. (2009a). Herpes simplex virus type1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* 217, 131–138. doi: 10.1002/path.2449

Wozniak, M. A., Frost, A. L., and Itzhaki, R. F. (2009b). Alzheimer's disease specific tau phosphorylation is induced by herpes simplex virus type 1. *J. Alzheimers Dis.* 16, 341–350. doi: 10.3233/JAD-2009-0963

Wozniak, M. A., Frost, A. L., Preston, C. M., and Itzhaki, R. F. (2011). Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with herpes simplex virus type 1. *PLoS ONE* 6:e25152. doi: 10.1371/journal.pone.0025152

Wozniak, M. A., Itzhaki, R. F., Shipley, S. J., and Dobson, C. B. (2007). Herpes simplex virus infection causes cellular β-amyloid accumulation and secretase upregulation. *Neurosci. Lett.* 429, 95–100. doi: 10.1016/j.neulet.2007.09.077

Wu, H. M., Huang, C. C., Chen, S. H., Liang, Y. C., Tsai, J. J., Hsieh, C. L., et al. (2003). Herpes simplex virus type 1 inoculation enhances hippocampal excitability and seizure susceptibility in mice. *Eur. J. Neurosci.* 18, 3294–3304. doi: 10.1111/j.1460-9568.2003.03075.x

Zambrano, A., Solis, L., Salvadores, N., Cortés, M., Lerchundi, R., and Otth, C. (2008). Neuronal cytoskeletal dynamic modification and neurodegeneration induced by infection with herpes simplex virus type 1. *J. Alzheimers Dis.* 14, 259–269.

Zhang, Y. W., Thompson, R., Zhang, H., and Xu, H. (2011). APP processing in Alzheimer's disease. *Mol. Brain* 4, 3. doi: 10.1186/1756-6606-4-3

Zheng, H., and Koo, E. H. (2006). The amyloid precursor protein: beyond amyloid. *Mol. Neurodegener.* 1, 5. doi: 10.1186/1750-1326-1-5

Ziaie, Z., Brinker, J. M., and Kefalides, N. A. (1994). Lithium chloride suppresses the synthesis of messenger RNA for infected cell protein-4 and viral deoxyribonucleic acid polymerase in herpes simplex virus-1 infected endothelial cells. *Lab. Invest.* 70, 29–38.

Ziaie, Z., and Kefalides, N. A. (1989). Lithium chloride restores host protein synthesis in herpes simplex virus-infected endothelial cells. *Biochem. Biophys. Res. Commun.* 160, 1073–1078. doi: 10.1016/S0006-291X(89)80112-3

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The battle of Alzheimer's Disease – the beginning of the future Unleashing the potential of academic discoveries

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Alzheimer's Disease (AD) is the most common form of dementia, affecting approximately 36 million people worldwide. To date there is no preventive or curative treatment available for AD, and in absence of major progress in therapeutic development, AD manifests a concrete socioeconomic threat. The awareness of the growing problem of AD is increasing, exemplified by the recent G8 Dementia Summit, a meeting held in order to set the stage and steer the compass for the future. Simultaneously, and paradoxically, we have seen key players in the pharmaceutical industry that have recently closed or significantly decreased their R&D spending on AD and other CNS disorders. Given the pressing need for new treatments in this area, other actors need to step-in and enter this drug discovery arena complementing the industrial efforts, in order to turn biological and technological progress into novel therapeutics. In this article, we present an example of a novel drug discovery initiative that in a non-profit setting, aims to integrate with both preclinical and clinical academic groups and pharmaceutical industry to explore the therapeutic potential of new concepts in patients, using novel biology, state of the art technologies and rapid concept testing.

Keywords: Alzheimer's disease, drug discovery and development, technology, pharmaceutical, non-profit, collaborative research

INTRODUCTION

During the last 25 years the population of the world has increased from 5.3 to almost 7.2 billion people (World Population Review, 2013). While an increased standard of living has resulted in decreased mortality, a global increased awareness of healthy living in combination with major progress in several areas of medicine has also made significant contribution to the increase in world population. Indeed, the death caused by heart disease, stroke, and cancer have decreased in the USA from 68% (1980) to 53% (2010) (75 Years of Mortality in the United States, 1935–2010, 2012). In parallel with these encouraging numbers, another major medical threat is emerging which could result in a major socioeconomic chaos in the absence of medical progress. The threat is named Alzheimer's Disease (AD). Worldwide, approximately 36 million people are diagnosed having dementia and 7.7 million new cases are discovered every year. AD is the most common cause of dementia among the elderly and may contribute up to 60–70% of all cases (World Health Organization, 2012). Currently there is no curative or preventive treatment for the patients and only a few drugs, which all provide limited symptomatic relief during a relatively short time frame, are available. Age is by far the largest risk factor for developing AD and the incidence increases exponentially from about 1% of 65-year old people to 30% of all 85-year-old people. With the current global increase in average lifespan, approximately 115 million people are estimated to be suffering from AD in 2050. Besides the tremendous suffering for the affected individuals and their close relatives, the cost for the society is estimated to be more than 200 billion USD in 2013 and 1200 billion USD by 2050 in the USA alone, a number that will put the healthcare system under enormous strain (Alzheimer's Association, 2013; World Alzheimer Report 2013, 2014).

Awareness, at the highest political level, of the emerging threat of dementia was highlighted with the recent G8 summit on dementia held in London in December 2013. The aim of the meeting was to develop a coordinated global action on dementia, and to shape an effective international response to dementia, resulting in the communication of a declaration (G8 Dementia Summit Agreements, 2013). This declaration included; "an ambition to identify a cure, or a disease-modifying therapy, for dementia by 2025." This will be achieved by, e.g., significantly increasing the amount spent on dementia research, increasing the number of people involved in clinical trials and studies on dementia as well as develop an international action plan for research. These efforts clearly state the sense of urgency in this field to move forward and for the research community to find a solution.

Dementia or senility as a concept or condition is a very old phenomenon, referred to in medical texts since antiquity. Today, we are aware of a large number of diseases that cause dementia but AD represents the most common one (Ballenger, 2006; Lane et al., 2012; Savonenko et al., 2012; Agis-Torres et al., 2014). A major milestone in the history of AD took place approximately 100 years ago (1907), when the German neuropsychiatrist Alois

Alzheimer first described the key neuropathological hallmarks of the disease (Hardy, 2006). During the examination of brain sections derived from his patient Auguste D, a middle-aged woman who suffered and died from dementia, Dr. Alzheimer discovered so-called senile plaques and neurofibrillary tangles in the brain parenchyma. These were seminal findings, linking for the first time the form of dementia now called "Alzheimer's disease," to specific pathological changes within the brain. However, the recognition that dementia was a result of abnormal pathological changes distinct from normal aging, did not really gain momentum until the 1970s, with the birth of the cholinergic hypothesis. A number of studies showed that, in AD brains, there was a particular loss of markers for cholinergic function as well as basal forebrain cholinergic neurons early on in the disease pathology (Francisa et al., 1999). These discoveries fueled an intense research effort to find drugs that could target cholinergic dysfunction and today three out of the four commonly prescribed drugs for AD (donepezil, rivastigmine and galantamine) are so-called cholinesterase inhibitors (Greenberg et al., 2013). These drugs inhibit the degradation of acetylcholine in the synaptic cleft, thereby sustaining the action of acetylcholine with a subsequent improvement in cognitive function in the patient. However, the underlying neurodegenerative cascade is not affected by these treatments, and the effect is thereby limited in time due to the continuous loss of cholinergic neurons and thus acetylcholine production in the disease. Moreover, gastrointestinal side effects, relating to muscarinic receptor engagement in the gut, as well as headache and effects on heart rate are also dose-limiting factors for the patients. The fourth described drug for AD is memantine, a weak NMDA receptor antagonist, prescribed for moderate to severe AD. Similar to the acetylcholinesterase inhibitors, memantine provides symptomatic relief for a limited time period, but is not believed to alter the neurodegenerative cascade of the disease.

ALZHEIMER'S DISEASE – NAVIGATION THROUGH COMPLEXITY

Although the progress in the drug development for AD has been limited over the last 30–40 years, significant progress has been made in understanding the mechanisms behind the development of AD, e.g., the pathology, neurobiology, and genetics (Corbette et al., 2012; Dunkel et al., 2012; Kepp, 2012; Greenberg et al., 2013). Among these finding were the discoveries of the amyloid beta peptide (Aβ) and the tau protein, the principal components of senile plaques and neurofibrillary tangles, respectively. Furthermore, researchers were also able to identify more than 200 disease causing mutations localized to three genes directly involved in Aβ generation as well as unravel some of the molecular machinery and underlying mechanisms of Aβ production. Combined, these discoveries provided a strong rationale for a better understanding of the neurotoxic role of Aβ in AD and for the development of Aβ amyloid-directed therapies. Accordingly, during the last 15 years, both active and passive vaccines targeting Aβ as well as several inhibitors and modulators targeting the aggregation or synthesis of Aβ peptide has been developed and tested in AD clinical trials. In addition, a large number of other drug candidates exhibiting a range of different non-Aβ targeting therapeutic mechanism of action have been tested clinically. These include those that are primarily aiming for a symptomatic effect by increasing synaptic activity, e.g., compounds that interact with nicotinic, histaminergic, and serotonergic receptors (Misra and Medhi, 2013). Despite that many of these drug candidates have shown promising effect in preclinical models, the results from clinical trials have been disappointing, and no new drugs after the acetylcholine esterase inhibitors and the NMDA blocker memantine have made it all the way to the market. It is well recognized that drug discovery in the AD field has been hampered by the failure of preclinical models to recapitulate some of the key features of the AD pathogenesis (Savonenko et al., 2012). The use of these models to unravel the complex features of the neurodegenerative cascade in AD, as well as translational tools in, e.g., dose prediction studies, have therefore been limited.

The reasons for the clinical failures are likely many, and differ among the drug candidates tested (Mangialasche et al., 2010; Schneider et al., 2014). Major contributors to the general lack of success include insufficient target exposure to achieve a clinically meaningful effect, that the drug was not tested long enough, safety issues due to on- and off-target pharmacology and/or that the disease context was not appropriate for the therapeutic mechanism explored (see **Box 1**). The latter stems from recent progress in biomarker research, which has shown that the AD pathological cascade start early and takes place over decades prior to symptoms onset, and where Aβ amyloidosis dominates at early stages followed by more overt and outbread neuronal dysfunction and neuronal degeneration, implicating other pathogenic drivers of disease, once the disease becomes symptomatic and progresses into severe stages of dementia (Sperling et al., 2011). Therefore, a therapy tailored for a specific component of the AD pathogenic cascade may have to be given to patients at a specific stage of the disease in order to be efficacious. The recent development of specific radiopharmaceutical diagnostic tools for PET imaging

BOX 1 | Some key challenges to be addressed in the development of novel therapeutics for Alzheimer's disease.

- Identify and link novel pathways and biomarkers to AD progression.
- Improved preclinical modeling of disease relevant pathology.
- Engaging multiple targets might be required (polypharmacology) and thereby using a multi-drug regimen.
- Establish and conduct conclusive proof-of-concept (PoC) studies.
- Use appropriate patient inclusion criteria in clinical trials.
- Achieve sufficient CNS exposure/target engagement of the drug.
- Establish appropriate biomarkers and/or surrogate markers for target engagement.
- Establish surrogate markers for clinical effect to shortened the length of clinical trial.
- Improved clinical endpoints, e.g., more sensitive and relevant cognitive measures.
- Avoid safety issues due to chronic, systemic exposure of the drug.
- Feedback to discovery from clinical PoC to refine hypothesis and improve research models.
- Handle potential drug–drug interactions (patient population often on other medications).

Aβ-amyloid plaque density (e.g., florbetapir and florbetaben) has been extremely useful in assessing amyloid load in patients at various stages of the disease (Herholz and Ebmeier, 2011). Thanks to these new methods with improved resolution combined with improvements in biomarker monitoring, the characterization and diagnosis of patients suspected to be suffering from AD pathogenesis have improved. Interestingly, such analyses have revealed that as many as 25–30% of the patients included in recent trials with therapeutic antibodies targeting Aβ amyloidosis were in fact negative for Aβ-amyloid in their brains (Doody et al., 2014; Salloway et al., 2014). These observations highlight the need to design trials where a lot of emphasis is put on the patient inclusion criteria in order to increase the odds to get meaningful clinical response of the therapeutic compound and/or mechanism tested.

FUTURE IDEAS TOWARDS NOVEL AD THERAPEUTICS

THE POTENTIAL OF ACADEMIC DISCOVERIES IN AD

The pharmaceutical industry (pharma) is currently under pressure from a range of different challenges in its environment, e.g., increased R&D costs, patent cliffs causing a major loss of income, high attrition of projects in the portfolio, increasing cost-constraints in the healthcare system mandating generics and parallel import, and not the least, more demanding regulatory requirements (Kola and Landis, 2004; Cuatrecasas, 2006; Schachter and Ramoni, 2007; Munos, 2009; Paul et al., 2010; Scannell et al., 2012; Slusher et al., 2013). In our view, an attractive alternative to the current way of working, as well as a way to offer a potential solution to the gaps in drug discovery and development pipelines, is to increase the research efforts in non-profit biopharmaceutical research institutions, such as universities and private research institutes, where creative and innovative science is not severely restricted by commercial objectives, but where novel hypothesis can be tried with industrial stringency in a dedicated way. These types of smaller research units could make significant contributions to the field since it is well recognized that small- and medium-sized biotechnological companies have been more successful than big pharmaceutical companies in moving candidate substances through the pipeline, and in particular, more successful in producing biological products, such as monoclonal antibodies, vaccines, and peptides (Munos, 2009). Non-profit organizations are well suited to address areas of large unmet need in rare CNS-disorders classified as orphan diseases. These diseases are, on the whole, not commercially blockbusters and, thus, research funded by philanthropists and public sources will be key to encourage work in these areas. It is also likely that certain therapeutics developed for an orphan indication may turn out to be efficacious in related disorders that share certain common features or principles of pathogenesis. The availability of clinically validated drugs is extremely valuable, and repositioning of existing drugs for novel indications opens up novel exciting possibilities to accelerate therapeutic development in complex CNS disorders such as AD.

Today when many major pharmaceutical companies are downsizing their R&D efforts in the CNS area (Alzheimer's Drug Failure: implication for Future R&D in Neuroscience, 2012), they have a strong need to get access to outside expertise in specific scientific areas such as for the identification of novel targets, increasing the knowledge in certain target families, advancing new platform technologies for preclinical and clinical research, improving the diagnosis of disease, pharmacology and safety biomarker development, access to human cells and tissues for target validation, as well as how to perform earlier proof-of-mechanism (PoM) and proof-of-concept (PoC) studies, including clinical safety studies. Some of this will of course be possible through established collaborations between the pharmaceutical industry and third parties such as contract research organization (CRO) and academia, such an example is the Dominantly Inherited Alzheimer Network (DIAN, established in 2008), which test diagnostics and novel treatments in a specific patient population. Although many CROs now provide customers with various non-regulatory *in vitro* and *in vivo* experiments during early discovery, their main activities are regulatory-driven (in accordance with good laboratory practice (GLP) in later phases of drug development). However, we see an attractive alternative in non-profit biomedical research institutions, where scientific collaborations can be based on a close interaction rather than contracting. This collaborative network/partnership will facilitate a closer preclinical-to-clinical translation at the target, model, assay, substance, patient and clinical testing levels as well as in the development of new technologies. Innovation and progress driven in these areas by such constellations will certainly accelerate therapeutic development in AD, and thereby attract increased interest from the big pharmaceutical companies, given that the potential return of investment would be substantial.

AlzeCure FOUNDATION – AN EXAMPLE

AlzeCure is a Swedish-based non-profit drug discovery foundation, whose aim is to develop novel therapeutics for AD and related disorders (see **Box 2** and **Figure 1**). This new organization was founded in 2013 by former AstraZeneca scientists with more than 30 years of experience from the CNS drug discovery and development area. The team exhibits complementary skills and expertise, from medicinal chemistry, screening, *in vivo* translational/biomarker competence to ADME and clinical development. The strategy of AlzeCure Foundation ("AlzeCure") is to use its wide experience and international network in the Alzheimer field to run collaborative projects with external expertise that has the potential to deliver real value for the patients. The team focuses on areas of research and development, which are of urgent need of innovative solutions, such as mechanistic diversity, preclinical models, drug delivery, clinical testing, and biomarker monitoring. We are convinced that rapid feedback to the drug discovery process from positive or failed PoC studies are essential in order to improve our understanding and to progress the development of efficacious therapeutics. Such backtranslation is also crucial in order to accelerate the development of follow-on compounds that address the same mechanism, but have improved drug-like characteristics, or to identify related mechanistic targets that will lead to new clinical studies. History has proven that there is a synergy between technology and biology innovation and in that interface, exciting discoveries take place that can result in novel products with a remarkable potential. Similarly, we strongly believe that technology breakthroughs will contribute significantly to speed up and improve the development of novel therapies in major CNS disorders.

BOX 2 | Strategies of the AlzeCure foundation.

Strong focus on both innovative drug discovery and clinical testing.
Use unique technology for drug administration.
Develop novel technology for biomarker monitoring.
Explore new therapeutic mechanisms and principles.
Develop of combination treatments affecting more than one biological pathway.
Test old marketed drugs or early closed candidate drugs on novel AD-targets.
Run phenotypic screens with well-defined pathological endpoints.
Collaborate with academic and industrial expertise to complement in-house expertise and capabilities.
Design early PoM and PoC clinical studies to test new hypothesis.
Optimize patient selection for earlier clinical safety and efficacy studies, based on qualified clinical biomarkers.

The laboratories of AlzeCure are located in direct vicinity to biotech companies and Karolinska Institute Alzheimer Research Center with academic groups of leading experts in neurobiology, preclinical and clinical laboratories with a long track record in AD translational research and in clinical trials. The strategic localization of AlzeCure has resulted in face-to-face interactions with these institutions and disciplines on a regular basis, which is a strong enabling factor for effective project progression and success. The non-profit nature of AlzeCure has turned-out to facilitate the establishment of joint efforts with both academia and industry and attract alternative funding. Today, AlzeCure manages several collaborations with international academic experts in both preclinical and clinical research. AlzeCure collaborates with several biotech companies, which provide unique chemistry and technology that in combination with neuronal screening assays based on induced pluripotent stem cells (iPS) and phenotypic endpoints, provide the basis for novel drug discovery. The most advanced collaboration aims at a novel way of administering therapeutic tau-directed antibodies to the brain, in order to increase target engagement and thereby therapeutic activity[1]. Tau is emerging as a new important molecular target in AD, and several anti-tau antibodies are rapidly approaching clinical trials. However, a major challenge with peripheral administration of antibodies is to reach the target in the brain to a sufficient extent. AlzeCure is together with NsGene, a Denmark and US-based biotechnology company, and Peter Davies (Feinstein Institute) evaluating a new concept to deliver therapeutic antibodies to the brain, using a novel proprietary Brain-Repair technology platform developed by NsGene (Lindvall and Wahlberg, 2008). The Brain-Repair platform is an implant comprised a semi-permeable hollow fiber containing a human cell line, which is engineered to locally produce therapeutic proteins of choice that diffuse into the target tissue. The implant has been clinically validated in patients diagnosed with AD through collaboration between NsGene and the Karolinska Institute (Eriksdotter-Jönhagen et al., 2012). An opportunity with this device would be to more rapidly test the hypothesis in a PoC study in a well-defined tauopathy patient population for an orphan indication, before entering large expensive phase III trials. In this manner, we foresee getting both the technology, therapeutic mode of action, clinical effect, and safety evaluated, in an effective and conclusive manner within a shorter time frame compared to common standards of clinical testing in AD. Obviously the Brain-Repair implant also offers a very exciting concept to rapidly test other therapeutic hypothesis beyond tau-directed antibodies, and illustrates a concrete example where technology innovation together with new biology form a novel avenue for therapy development applicable for multiple CNS disorders.

A co-initiator and also major financial contributor to AlzeCure is the Swedish Alzheimer foundation, a charity organization. This type of direct financial commitment and interest in drug discovery and development in complex disorders such as CNS diseases from charity organizations is novel and may point to a new path for future organizations. In addition, the Swedish Brain Power program is sponsoring AlzeCure with two Ph.D. positions, which are highly integrated into the academic labs thereby providing the basis for a strong two-way communication between academic science and industrial development, which is of high mutual interest. Given the declining interest from many big pharmaceutical companies and venture capitalists alike in CNS disorders and the pressing need for new medications, initiatives like these are new ways of trying to directly stimulate drug discovery efforts in an area of great medical need. Interestingly, in line with the intention of AlzeCure, Alzheimer Research UK announced at the end of 2013 that they were to initiate a Drug Discovery Institute consisting of leading academic groups in the UK that will have close access to both preclinical and clinical research units and hospitals specialized in neurodegenerative diseases that cause dementia.

CONCLUSION

The problem we as a society are facing is of unseen proportions, highlighting the need of concerted actions at multiple levels of society. A firm political leadership paired with a well-designed regulatory framework and strong incentives for academia, the industry and the health care providers is needed in order to consolidate the resources and optimize the process required to make rapid and significant progress in therapeutic development for AD. In this light it may seem paradoxical that several major industrial actors, such as AstraZeneca, Bristol-Myers Squibb, GlaxoSmithKline, and Novartis have either abandoned the neuroscience therapeutic indication as such, or heavily decreased their head count within this area (Abbot, 2011). However, other companies, e.g., Eli Lilly, Roche, Pfizer have continued a strong interest in the area and are closely collaborating the regulatory agencies, which recently resulted in a Guidance for Industry from FDA on Alzheimer's disease: Developing drugs for the treatment of early stage disease (U.S. Food and Drug Administration, 2013). The medical need is however still unmet and rapidly growing, thus something radical needs to be done. For a start, we believe that new initiatives such as the AlzeCure Foundation and Drug Discovery Alzheimer Research UK group, both being non-profit and

[1] See www.alzecure.org

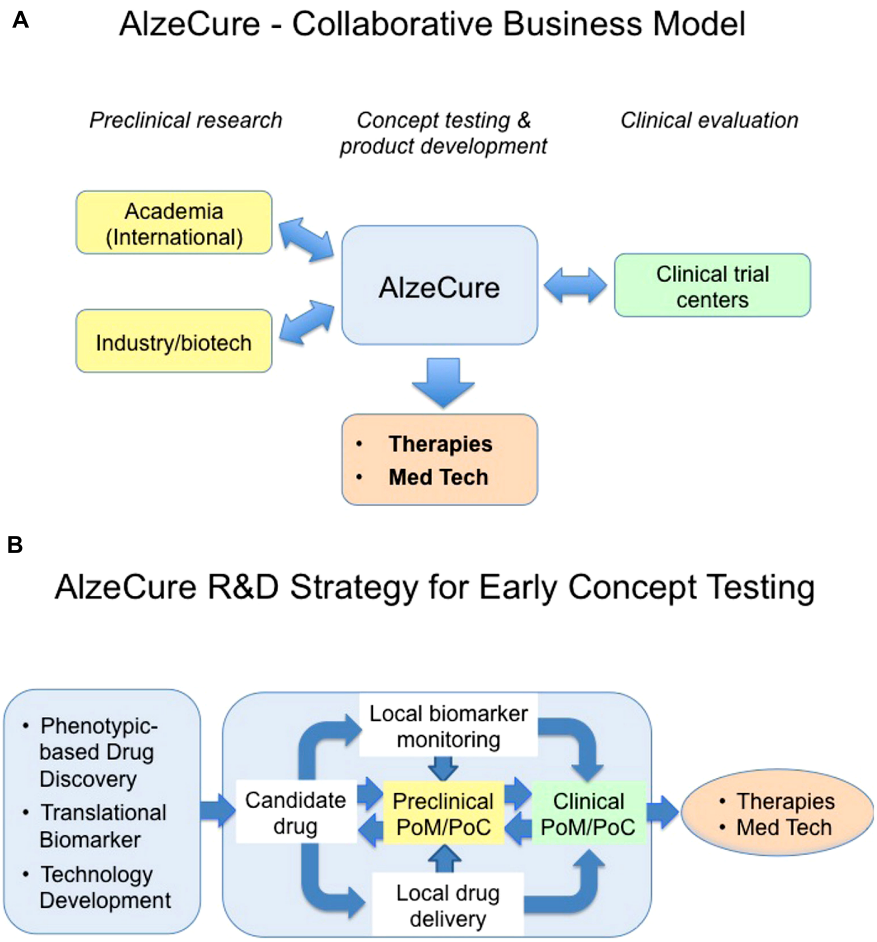

FIGURE 1 | This cartoon illustrates the proposed AlzeCure business (A) and strategy models (B) to develop new therapies and medicinal technology products, highlighting the proof-of-mechanism (PoM)/proof-of-concept (PoC) as an important milestone in drug discovery and development process. Traditionally, in order to reach clinical PoM and PoC with "standard peripherally administered" drug candidates in a chronic condition, the drug needs to display key and optimized features including adsorption, distribution, metabolism, tolerability, and BBB permeability, in addition to its desired pharmacology and safety. This is seldom achieved despite significant medicinal chemistry, an effort that takes place over a very long time in the discovery phase. As a consequence, many suboptimal compounds are being explored in the clinic without being able to test the hypothesis they were aimed for in a conclusive manner. Building the need to get strong evidence for PoM/PoC, including close collaboration with international academic preclinical research, utilizing new Translational enabling technologies into the discovery process, is crucial. To reduce the attrition due to lack of efficacy and safety, it is important to design the first clinical trials, a design based on appropriate animal models, pharmacokinetic/pharmacodynamic modeling, and utilizing biomarkers and surrogate markers, in the "right patients," to facilitate that the molecular target is being hit at an expected concentration to give the anticipated physiological response without safety issues. Optimally, the PoM/PoC clinical trials are conducted at Clinical Trial Centers that are closely involved in the discovery research. It is also important to have a rapid feed-back to the drug discovery process from positive or failed PoM/PoC studies to improve the understanding of mechanisms, and to accelerate the development of follow-up compounds that address the same mechanism, but with improved drug-like characteristics or to identify related mechanistic targets that will lead to new clinical studies.

taking full advantage of the open-minded academic style of working with the stringent and goal-oriented industrial R&D, have the promise of being more innovative and effective unleashing the potential of academic discoveries and providing an exciting new framework for the long-term objective to deliver effective therapies to the benefit of the patients.

REFERENCES

Abbot, A. (2011). Novartis to shut brain research facility. *Nature* 480, 161–162.

Agis-Torres, A., Sölhuber, M., Fernandez, M., and Sanchez-Montero, J. M. (2014). Multi-target-directed ligands and other therapeutic strategies in the search of a real solution for Alzheimer's disease. *Curr. Neuropharmacol.* 12, 2–36. doi: 10.2174/1570159X113116660047

Alzheimer's Association. (2013). *Alzheimer's Disease Facts and Figures.* Chicago, IL: Alzheimer's Association. Available at: http://www.alz.org/alzheimers_disease_facts_and_figures.asp

Alzheimer's Drug Failure: implication for Future R&D in Neuroscience. (2012). *Today's geriatric medicine.* Available at: http://todaysgeriatricmedicine.com/news/081412_news.shtml

Ballenger, J. F. (2006). Progress in the history of Alzheimer's disease: the importance of context. *J. Alzheimer's Dis.* 9, 5–13.

Corbette, A., Pickett, J., Burns, A., Corcoran, J., Dunnett, S. B., Edison, P., et al. (2012). Drug repositioning for Alzheimer's disease. *Nat. Rev. Drug Discov.* 11, 833–846. doi: 10.1038/nrd3869

Cuatrecasas, P. (2006). Drug discovery in jeopardy. *J. Clin. Invest.* 116, 2837–2842. doi: 10.1172/JCI29999

Doody, R. S., Thomas, R. G., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., et al. (2014). Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N. Engl. J. Med.* 370, 311–321. doi: 10.1056/NEJMoa1312889

Dunkel, P., Chai, C. L. L., Sperlagh, B., Hulett, P. B., and Matyus, P. (2012). Clinical utility of neuroprotective agents in neurodegenerative diseases: current status of drug development for Alzheimer's, Parkinson's and Huntington's diseases, and amyotrophic lateral sclerosis. *Expert Opin. Invest. Drugs* 21, 1267–1308. doi: 10.1517/13543784.2012.703178

Eriksdotter-Jönhagen, M., Linderoth, B., Lind, G., Aladellie, L., Almkvist, O., Andreasen, N. K., et al. (2012). Encapsulated cell biodelivery of nerve growth factor to the basal forebrain in patients with Alzheimer's disease. *Dement. Geriatr. Cogn. Disord.* 33, 18–28. doi: 10.1159/000336051

Francisa, P. T., Palmerb, A. M., Snapeb, M., and Wilcockc, G. K. (1999). The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J. Neurol. Neurosurg. Psychiatry* 66, 137–147. doi: 10.1136/jnnp.66.2.137

G8 Dementia Summit Agreements. (2013). Department of Health and Prime Minister's Office. London, UK. Available at: https://www.gov.uk/government/publications/g8-dementia-summit-agreements doi: 10.1016/S0140-6736(07)61487-1

Greenberg, B. D., Carrillo, M. C., Ryan, J. M., Gold, M., Gallagher, K., Grundman, M., et al. (2013). Improving Alzheimer's disease phase II clinical trials. *Alzheimer's Demen.* 9, 39–49. doi: 10.1016/j.jalz.2012.02.002

Hardy, J. (2006). A hundred years of Alzheimer's disease research. *Neuron* 52, 3–13. doi: 10.1016/j.neuron.2006.09.016

Herholz, K., and Ebmeier, K. (2011). Clinical amyloid imaging in Alzheimer's disease. *Lancet Neurol.* 10, 667–670. doi: 10.1016/S1474-4422(11)70123-5

Kepp, K. K. (2012). Bioinorganic chemistry of Alzheimer's disease. *Chem. Rev.* 112, 5193–5239. doi: 10.1021/cr300009x

Kola, I., and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 3, 711–715. doi: 10.1038/nrd1470

Lane, R. F., Shineman, D. W., Steele, J. W., Lee, L. H., and Fillit, H. M. (2012) Beyond amyloid: the future of therapeutics for Alzheimer's disease. *Adv. Pharmacol.* 64, 213–271. doi: 10.1016/B978-0-12-394816-8.00007-6

Lindvall, O., and Wahlberg, L. U. (2008). Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease? *Exp. Neurol.* 209, 82–88. doi: 10.1016/j.expneurol.20008.019

Mangialasche, F., Solomon, A., Winblad, B., Mecocci, P., and Kivipelto, M. (2010). Alzheimer's disease: clinical trials and drug development. *Lancet Neurol.* 9, 702–716. doi: 10.1016/S1474-4422(10)70119-8

Misra, S., and Medhi, B. (2013). Drug development status for Alzheimer's disease: present scenario. *Neurol. Sci.* 34, 831–839. doi: 10.1007/s10072-013-1316-x

Munos, B. (2009). Lessons from 60 years of pharmaceutical innovation. *Nat. Rev. Drug Discov.* 8, 959–968. doi: 10.1038/nrd2961

Paul, S. M., Mytelka, D. S., Dunwiddie, C. T., Persinger, C. C., Munos, B. H., Lindborg, S. R., et al. (2010). How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discov.* 9, 203–214. doi: 10.1038/nrd3078

Salloway, S., Sperling, R., Fox, N. C., Blennow, K., Klunk, W., Raskind, M., et al. (2014). Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N. Engl. J. Med.* 370, 322–333. doi: 10.1056/NEJMoa1304839

Savonenko, A. V., Melnikova, T., Hiatt, A., Li, T., Worley, P. F., Troncoso, P. F., et al. (2012). Alzheimer's therapeutics: translation of preclinical science to clinical drug development. *Neuropsychopharmacol. Rev.* 37, 261–277. doi: 10.1038/npp.2011.211

Scannell, J. W., Blanckley, A., Boldon, H., and Warrington, B. (2012). Diagnosing the decline in pharmaceutical R&D efficiency. *Nat. Rev. Drug Discov.* 11, 191–200. doi: 10.1038/nrd3681

Schachter, A. D., and Ramoni, M. F. (2007). Clinical forecasting in drug development. *Nat. Rev. Drug Discov.* 6, 107–108. doi: 10.1038/nrd2246

Schneider, L. S., Mangialasche, F., Andreasen, N., Feldman, H., Giacobini, E., Jones, R., et al. (2014). Alzheimer's disease clinical trials and late-stage drug development: a retrospective appraisal. *J. Intern. Med.* 275, 251–283. doi: 10.1111/joim.12191

Slusher, B. S., Conn, P. J., Frye, S., Glicksman, M., and Arkin, M. (2013). Bringing together the academic drug discovery community. *Nat. Rev. Drug Discov.* 12, 811–812. doi: 10.1038/nrd4155

Sperling, R. A., Aisen, P. S., Beckett, L. A., Bennett, D. A., Craft, S., Fagan, A. M., et al. (2011). Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's Dement.* 7, 280–292. doi: 10.1016/j.jalz.2011.03.003

The Dominantly Inherited Alzheimer Network (DIAN). St. Louis, MO: Washington University. Available at: http://www.dian-info.org/default.htm

U.S. Food and Drug Administration. (2013). *Guidance for Industry: Alzheimer's Disease: Developing Drugs in the Treatment of Early Stage Disease.* Rockville, MD: FDA.

World Alzheimer Report 2013. (2014). Alzheimer's Disease International. London, UK. Available at: http://www.alz.co.uk/research/world-report-2013

75 Years of Mortality in the United States, 1935–2010. (2012). Centers for Disease Control and Prevention, National Center for Health Statistics. Hyattsville, MD. Available at: http://www.cdc.gov/nchs/data/databriefs/db88.htm

World Health Organization. (2012). *Dementia Fact Sheet No. 362 April 2012.* Available at: http://www.who.int/mediacentre/factsheets/fs362/en/

World Population Review. (2013). Available at: http://worldpopulationreview.com/continents/world-population/ (accessed March 15, 2014).

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Sirtuin modulators control reactive gliosis in an *in vitro* model of Alzheimer's disease

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Among neurodegenerative disorders, Alzheimer's disease (AD) represents the most common cause of dementia in the elderly. Several genetic and environmental factors have been identified; however, aging represents the most important risk factor in the development of AD. To date, no effective treatments to prevent or slow this dementia are available. Sirtuins (SIRTs) are a family of NAD^+-dependent enzymes, implicated in the control of a variety of biological processes that have the potential to modulate neurodegeneration. Here we tested the hypothesis that activation of SIRT1 or inhibition of SIRT2 would prevent reactive gliosis which is considered one of the most important hallmark of AD. Primary rat astrocytes were activated with beta amyloid 1-42 (Aβ 1-42) and treated with resveratrol (RSV) or AGK-2, a SIRT1 activator and a SIRT2-selective inhibitor, respectively. Results showed that both RSV and AGK-2 were able to reduce astrocyte activation as well as the production of pro-inflammatory mediators. These data disclose novel findings about the therapeutic potential of SIRT modulators, and suggest novel strategies for AD treatment.

Keywords: resveratrol, AGK-2, sirtuins, beta-amyloid, astrocyte, reactive gliosis, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) represents one of the major health concern and it is a research priority since there is a pressing need to develop new agents to prevent or treat it. A part of the progressive deposition of beta amyloid peptide (Aβ) and accumulation of phosphorylated tau, several other alterations occur in AD brain, all concurring to neuronal loss. Among these, growing interest has been attracted by the role of inflammation in the onset and progression of this disorder. In fact, senile plaques and neurofibrillary tangles (which are considered the more characteristic hallmarks of AD) co-localize with activated astrocytes, suggesting for these cells a key role in the pathogenesis of AD (Meda et al., 2001; Craft et al., 2006). Along this line, in several experimental models it has been demonstrated that Aβ peptide fragments markedly alter astrocytes functions. This process is accompanied with a noticeable neuroinflammatory response, accounting for the synthesis of different cytokines and pro-inflammatory mediators which amplify neuropathological damage (Mrak and Griffin, 2001; Caricasole et al., 2003; Tuppo and Arias, 2005; Griffin, 2006; Scuderi et al., 2011, 2012, 2013). It is established that neuroinflammation is directly linked to neural dysfunction and cell death, representing a primary cause of neurodegeneration (Block and Hong, 2005). In fact, over-release of pro-inflammatory cytokines by glia cells causes neuronal dysfunction and loss of synapses, which correlates with memory decline. These phenomena are believed to precede neuronal death. Thus, research focused on developing therapeutic strategies directed at controlling the prolonged and uncontrolled glia activation should be encouraged.

An uncommon opportunity to improve inflammation and neurodegeneration simultaneously is provided by compounds able to modulate histone acetylation/deacetylation, since they participate in brain immune control and neuroprotection, in addition to their well-known effects on the molecular mechanisms associated to senescence and metabolic syndromes. Mounting evidence indicates sirtuins (SIRTs) exert neuroprotective effects in several models of neurodegeneration (Outeiro et al., 2008; Tang and Chua, 2008; de Oliveira et al., 2010). SIRTs, a family of NAD^+-dependent enzymes with seven isoforms identified (SIRT1-7), are implicated in the control of a variety of biological processes including transcriptional silencing, chromosomal stability, cell cycle progression, apoptosis, autophagy, metabolism, growth suppression, inflammation, and stress response (Gan and Mucke, 2008; Haigis and Sinclair, 2010).

Recent observations indicate both SIRT1 and SIRT2 regulate neuronal survival, but with divergent functional outcomes. Indeed, activation of SIRT1 mainly exerts neuroprotective actions, while SIRT2 fosters neurodegeneration. The reason for such opposite effect may be due to their different sub-cellular localization, which gives SIRT1 and SIRT2 distinct molecular targets (Harting and Knöll, 2010). It has been demonstrated that the overexpression of SIRT1 prevents neuronal death in tissue culture models of AD, amyotrophic lateral sclerosis, and polyglutamine toxicity, and it reduces hippocampal degeneration in a mouse model of AD (Kim et al., 2007; Li et al., 2007). Moreover, treatment with resveratrol (RSV), a polyphenolic compound acting as a pharmacological activator of SIRT1, is protective in a number

of experimental neurodegeneration paradigms (Anekonda, 2006; Sun et al., 2010). Resveratrol, like other polyphenol compounds including curcumin, displays a plethora of actions, behaving as a potent antioxidant agent, increasing SUMOylation, and activating protein kinase C, all mechanisms able to counteract astrocyte reactivity and protect neurons (Jefremov et al., 2007; Hoppe et al., 2013; Menard et al., 2013). Finally, it has been observed that both SIRT1 overexpression and RSV treatment are able to significantly decrease the Aβ-induced activation of NF-κB, thus operating a simultaneous control on both neurodegeneration and neuroinflammation processes (Chen et al., 2005). Indeed, NF-κB is a transcription factor which controls the expression of gene products involved in key cellular signaling, including those associated to inflammatory and degenerative events. Post-mortem studies on cerebral cortices from AD patients have established a correlation between loss of SIRT1 and the accumulation of Aβ and hyperphosphorylated tau proteins (Julien et al., 2009). Growing evidence indicates that also SIRT2 is involved in regulating several brain processes including oligodendrocyte mitosis and differentiation, cytoskeletal dynamics necessary for trafficking, neurite outgrowth and synaptic remodeling. Unlike SIRT1, SIRT2 appears to promote neuronal death. In fact, blocking SIRT2 counteracted alpha synuclein toxicity in Parkinson's disease models (Outeiro et al., 2007). However, less is known about the role of SIRT2 in AD.

On the basis of these considerations, we explored the effects of modulators of SIRTs on astrocyte activation and the subsequent inflammatory process. In particular our experiments focalized the ability of RSV, a SIRT1 activator, and AGK-2, a SIRT2-selective inhibitor, to control astrocyte activation and to suppress the production of pro-inflammatory mediators in primary rat astrocytes exposed to Aβ peptide. These findings suggest that either RSV or AGK-2 may be an effective agent for neurodegenerative diseases initiated or maintained by inflammatory processes.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENTS

Newborn Sprague-Dawley rats (1 or 2 days old) were used to obtain primary astroglial cultures (Vairano et al., 2002; Scuderi et al., 2011). Briefly, brain cortices were homogenized and processed to obtain single cells. Astrocytes were cultured at a density of 3 × 106 cells/75-cm^2 flask and incubated at 37°C in a humidified atmosphere containing 5% CO2. The culture medium used was DMEM supplemented with 5% inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Sigma–Aldrich, Milan, Italy), replaced 24 h after isolation and again one a week until astrocytes were grown to form a monolayer. Approximately 14–15 days after dissection, astrocytes were mechanically separated from microglia and oligodendrocytes. Obtained astrocytes were seeded onto 10-cm-diameter Petri dishes (1 × 106 cells/dish) or onto 24 well plates (1 x 105 cells/well). The monoclonal anti-glial fibrillary acidic protein (GFAP) was used to verify cell culture purity. Only cultures with more than 95% GFAP-positive cells were utilized. The 5% of non-astrocyte cells were microglia and oligodendrocytes.

All experiments were performed in accordance with the Italian Ministry of Health (DL 116/92), the Declaration of Helsinki, and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research, and they were approved by the Institutional Animal Care and Use Committee at our institution.

Mature astrocytes were challenged with 0.23 μM Aβ 1-42 (Tocris Bioscience, Bristol, UK) in the presence or absence of the following substances: RSV (2 – 10 – 50 μM), a well-known SIRT1 activator, or AGK-2 (0.35 – 3.5 – 35 μM), a potent SIRT2-selective inhibitor (both from Sigma–Aldrich). After 24 (for viability and protein expression analyses) or 72 h (for proliferation assay) of treatment, astrocytes were collected for experiments. The concentration of the substances was chosen according to literature (Howitz et al., 2003; Outeiro et al., 2007; Scuderi et al., 2011, 2012).

ANALYSIS OF ASTROCYTE VIABILITY BY NEUTRAL RED UPTAKE ASSAY

Astrocyte viability was evaluated 24 h after treatments by the neutral red uptake assay according to Repetto et al. (2008), with some modifications (Scuderi and Steardo, 2013). Cells were seeded in 24-well plates and treated as previously described. 24 h after treatments, the plates were incubated for 3 h at 37°C with a neutral red working solution (50 μg ml^{-1} in PBS 1X without calcium and magnesium, Sigma-Aldrich). The cells were washed and the dye removed from each well through a destain solution (ethanol:deionized water: glacial acetic acid, 50:49:1, v/v). The absorbance was read at 540 nm using a microplate spectrophotometer (Epoch, Bio Teck, Winooski, VT, USA). The values of treated cells were referred to control non-exposed cultures, and expressed as percentage variation.

ANALYSIS OF ASTROCYTE PROLIFERATION BY TRYPAN BLUE ASSAY

Trypan blue exclusion assay was performed to monitor astrocyte proliferation 72 h after treatments. This method is based on the principle that living cells do not take up the dye, whereas dead cells do. To determine the number of cells and their viability using trypan blue, 20 μl of trypsinized and re-suspended cells were mixed with 20 μl of 0.4% solution of trypan blue dye (Sigma–Aldrich) for 1 min. Cells were immediately counted using a Bürker chamber with a light microscope. All counts were done using four technical duplicates of each sample.

ANALYSIS OF PROTEIN EXPRESSION BY WESTERN BLOTTING

Western blot analyzes were performed on extracts of cell cultures challenged as previously described. 24 h after treatment, cells were detached from petri dishes and each pellet was suspended in ice-cold hypotonic lysis buffer containing NaCl 150 mM; Tris/HCl pH 7.5 50 mM; Triton X-100 1%; ethylenediaminetetraacetic acid [EDTA] 1 mM, supplemented with PMSF 1 mM, Aprotinin 10 μg/ml, Leupeptin 0,1 mM (Roche, Mannheim, Germany). After incubation for 40 min at +4°C, homogenates were centrifuged at 14000 rpm for 15 min and the supernatant removed and stored in aliquots at −80°C until use. Equivalent amounts (70 μg) of each sample calculated by Bradford assay were resolved on 12% acrylamide SDS-PAGE precast gels (Bio-Rad Laboratories). Proteins were transferred onto nitro-cellulose. Membranes were blocked with 5% wt/vol no-fat dry milk powder in Tris-buffered saline-Tween 0,1% (TBS-T) for 1 h before

overnight incubation at 4°C with one of the following primary antibodies: rabbit anti-GFAP (1:50000, Abcam plc, Cambridge, UK), rabbit anti-S100B (1:1000, Epitomics, Burlingame, CA, USA), rabbit anti-inducible nitric oxide synthase (iNOS; 1:9000, Sigma–Aldrich), rabbit anti-cyclooxygenase-2 (COX-2; 1:1000, Cell Signaling Technology, MA, USA), rabbit anti-β-actin (1:1500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After being extensively washed in TBS-T, membranes were incubated for 1 h at 25 °C with the secondary horseradish peroxidase-conjugated antibody (HRP conjugated goat anti-rabbit IgG, 1:30000, Jackson Immunoresearch Europe, Suffolk, UK). The immunocomplexes were visualized using an ECL kit (Amersham, Bucks, UK). Protein expression was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad laboratories) and a computer program (ImageJ software v1.44p, NIH, USA).

STATISTICAL ANALYSIS

Analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data were analyzed by one way analysis of variance (ANOVA) to determine statistical differences between experimental groups. Multiple comparisons were performed with Bonferroni's test for *post hoc* analyzes. Differences between mean values were considered statistically significant when $p < 0.05$.

RESULTS

EFFECT OF RSV AND AGK-2 ON ASTROCYTE VIABILITY AND PROLIFERATION.

First of all, we decided to perform experiments to assess the effect of the SIRT modulators on astrocyte viability and proliferation after Aβ 1-42 challenge. In fact, it has been already demonstrated that Aβ peptides are able to affect cell viability and to induce astrocyte proliferation (Allaman et al., 2010; Scuderi et al., 2012). Our results highlighted a significant increase in cell viability after 24 h treatment with Aβ 1-42 (**Figures 1A,C**, $p < 0.01$). RSV and AGK-2 were able to reduce this effect at the two higher concentrations used (**Figures 1A,C**). In addition, we found a reduction in cell viability after treatment with AGK-2 at the concentration of 35 μM on un-stimulated cells, indicating a cytotoxic effect (**Figure 1C**). Trypan blue experiments revealed a significant astrocyte proliferation after 72 h treatment with Aβ 1-42 (**Figures 1B,D**, $p < 0.01$). Once again, both RSV and AGK-2 significantly controlled such increase at the two higher concentrations used. Surprisingly, RSV 50 μM and AGK-2 35 μM caused a reduction in proliferation rate also in un-challenged astrocytes (**Figures 1B,D**).

EFFECT OF RSV AND AGK-2 ON ASTROCYTE ACTIVATION

In order to test the effect of RSV and AGK-2 on Aβ-induced astrogliosis, the expression of GFAP and S100B, specific markers of astrocyte activity, was explored. Reactive astrocytes display hypertrophied cell bodies and thickened processes exhibiting GFAP-immunoreactivity (O'Callaghan and Sriram, 2005; Olabarria et al., 2010). Using Western blot analysis, we observed a marked increase in the expression of GFAP after Aβ 1-42 challenge ($p < 0.01$; **Figure 2**). RSV was able to significantly attenuate such increase in a concentration dependent manner (**Figures 2A,B**). Likewise, the Aβ-induced GFAP overexpression was counteracted by AGK-2 at the three concentrations used (**Figures 2C,D**).

Similarly, the expression of S100B was investigated by Western blot. S100B is an astroglia-derived protein which acts as a neurotrophic factor and neuronal survival protein, even though the overproduction of S100B by activated astrocytes lead to further neurodegeneration. Elevated S100B levels are generally associated with a sustained reactive gliosis (Griffin, 2006; Donato and Heizmann, 2010). Results from cultured astrocytes showed a significant increase in S100B protein expression after Aβ 1-42 exposure ($p < 0.01$; **Figure 2**). Both RSV and AGK-2 controlled such increase. Also in this case, RSV exerted its effect in a concentration dependent manner (**Figures 2A,B**). Instead, all the AGK-2 concentrations completely abolished the Aβ-induced S100B increase (**Figures 2C,D**).

EFFECT OF RSV AND AGK-2 ON INFLAMMATION

Another set of experiments was aimed at assessing the effect of RSV and AGK-2 on the production of inflammatory factors induced by Aβ 1-42 challenge. In fact, astrocyte activation is linked to the production of pro-inflammatory mediators which, in turn, stimulate gliosis and can kill neighboring neurons (Mrak and Griffin, 2001; Ferreira et al., 2014). Treatment with Aβ 1-42 resulted in an increase in iNOS expression, as determined by Western blot analysis (**Figure 3**; $p < 0.05$). Interestingly, this observed effect was reduced by both RSV and AGK-2 at the two higher concentrations used (**Figures 3A–D**). Parallel results were obtained with immunoblot experiments aimed at studying COX-2 expression. In fact, Aβ 1-42 significantly increased COX-2 protein expression (**Figure 3**; $p < 0.05$). Also in this case, both RSV and AGK-2 significantly decreased such effect at the two higher concentrations used (**Figures 3A–D**).

DISCUSSION

The purpose of this study was to assess the efficacy of RSV, a well-known SIRT1 activator, and AGK-2, a potent SIRT2-selective inhibitor, in counteracting reactive gliosis, now considered one of the characteristic phenomena occurring in AD. AD leads to disability and death in a significant proportion of the world's aged population (Alzheimer's Association Report, 2013). However, the available treatments are limited and exert only symptomatic effects. Several promising drugs have recently failed to provide benefit, so there is urgent need to develop new, and hopefully more efficacious, drugs to affect AD course. To this attempt, in the last years researchers focused their attention on the role of reactive gliosis in the onset and progression of many neurodegenerative disorders, including AD. Produced results gave evidence that neuroinflammation and neurodegeneration mutually have a critical impact on AD course (Wyss-Coray and Rogers, 2012). For this reason, it is possible to assume that early combination of neuroprotective and anti-inflammatory treatments represents a particularly appropriate approach to AD (Di Filippo et al., 2008). Although neurodegenerative disorders have distinct clinical manifestations, many of the underlying pathogenic processes are similar (intra- or extracellular accumulation of misfolded proteins, cytoskeletal abnormalities, disruption of calcium homeostasis, mitochondrial dysfunction, and inflammation), and most of them are strongly

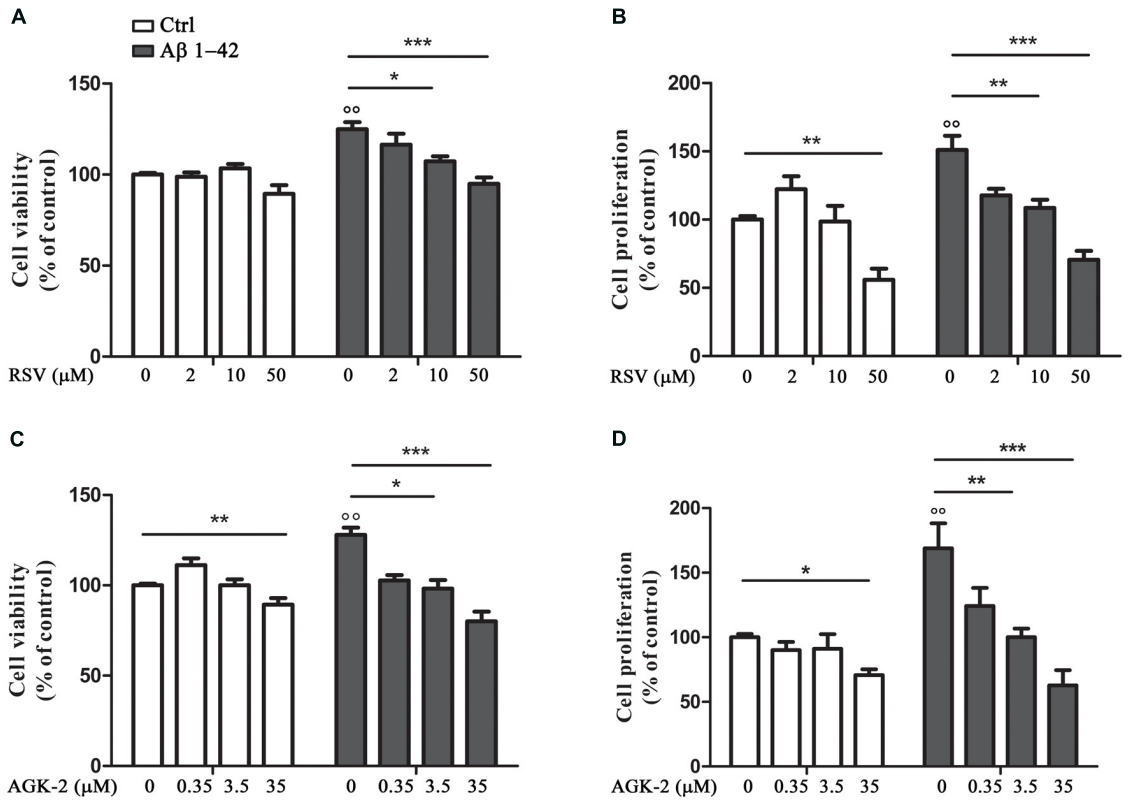

FIGURE 1 | Resveratrol (RSV) and AGK-2 affect astrocyte viability and proliferation induced by Aβ 1-42 challenge. Cells were challenged with 0.23 μM Aβ 1–42 in the presence or absence of one of the following substances: RSV (2 – 10 – 50 μM), a potent SIRT1 activator; AGK-2 (0.35 – 3.5 – 35 μM), a selective SIRT2 inhibitor. 24 h later cell viability was assessed by neutral red uptake assay **(A,C)**. 74 h after treatments cell proliferation was evaluated by trypan blu assay **(B,D)**. Results are expressed as cell viability-fold increase versus unchallenged (open bars) or Aβ-challenged cells (black bars). Results are the mean ± SEM of four experiments in triplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison test. $p < 0.01$ Aβ-challenged versus unchallenged cells; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ for multiple comparison among groups.

influenced by and increased during aging. In particular, in both early- and late-onset sporadic AD, aging represents a major contributing factor for the disease development and progression, although the precise role remains still unclear. Transcriptional profiling studies revealed that expression of genes that play central roles in synaptic plasticity, vesicular transport and mitochondria function is reduced, whereas expression of genes encoding for stress, inflammatory or immune factors is increased in aged human frontal cortex (Lu et al., 2004). These findings implicate ongoing DNA damage, oxidative stress and inflammation as contributors to the functional decline occurring in age-related neurodegenerative diseases, including AD.

In this context, the discovery of SIRTs, indicated as class III histone deacetylases (HDACs), offers a close relationship between aging, metabolism and neurodegeneration, thereby representing an innovative target to develop therapeutic strategies (Outeiro et al., 2008). SIRTs play pleiotropic biological functions that range from repression of gene expression (through histone deacetylation) to regulation of cellular differentiation and/or apoptotic processes, from control of energetic cell metabolism to that of aging events. These enzymes have been extensively studied because of their involvement in mediating the effect of caloric restriction (CR) in fostering longevity and healthy aging. In addition, many data indicate that SIRTs are potentially able to delay neurodegenerative diseases related to senescence, including AD. (Michan and Sinclair, 2007). It has been demonstrated that CR reduces the content of Aβ in the temporal cortex of squirrel monkeys, and such effect is inversely linked to SIRT1 expression in the same brain region (Qin et al., 2006a). Moreover, in a transgenic mouse model of AD, the same authors previously demonstrated that CR antagonizes Aβ neuropathology by increasing the SIRT1 and NAD^{+}/nicotinamide ratio (Qin et al., 2006b). Recently, SIRT2 inhibition has been proposed as a promising therapeutic strategy to achieve neuroprotection in *in vitro* and *in vivo* models of Parkinson's and Huntington's diseases (Outeiro et al., 2007; Luthi-Carter et al., 2010). Moreover, Spires-Jones et al. (2012) demonstrated that inhibition of SIRT2 is a safe and promising neuroprotective agent in both tau-associated frontotemporal dementia and AD.

It is recognized that Aβ affects cell viability and proliferation (Allaman et al., 2010; Scuderi et al., 2012). It is possible to speculate that these Aβ actions are due to its ability to enhance astrocyte metabolism turning on morpho-functional changes in such cells (Verkhratsky and Butt, 2007). Interestingly, our experiments highlighted alterations in astrocyte viability and proliferation after Aβ

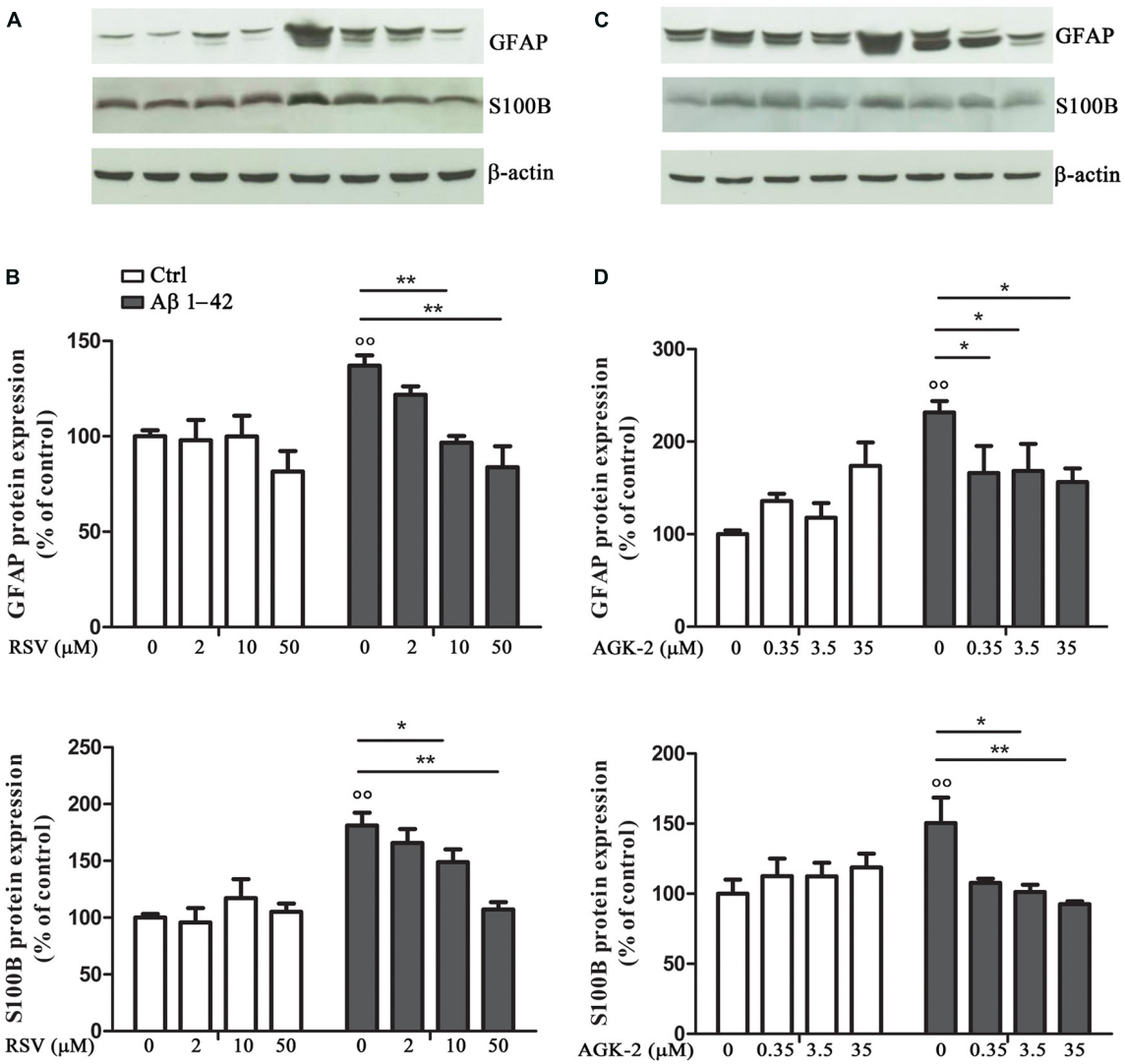

FIGURE 2 | Effect of RSV and AGK-2 on GFAP and S100B expression. 24 h after treatments, astrocytes were lysated and protein expression was evaluated. Representative Western blots for GFAP and S100B proteins in lysates from astrocytes challenged with Aβ 1-42 (0.23 μM) in the presence of RSV (2 – 10 – 50 μM; **A**) or AGK-2 (0.35 – 3.5 – 35 μM; **C**). Densitometric analyzes normalized to β-actin loading controls (**B,D** for RSV and AGK-2, respectively). Results are the mean ± SEM of four experiments in triplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison test. $p < 0.01$ Aβ-challenged versus unchallenged cells; $^{*}p < 0.05$; $^{**}p < 0.01$; for multiple comparison among groups.

1-42 challenge, and both RSV and AGK-2 markedly controlled these effects. SIRTs are considered as sensors of cell metabolic state because they finely modulate physiological processes. For this reason it is important to establish the appropriate concentrations to avoid dangerous unwanted consequences. In fact, in our conditions, we found that the highest concentrations used of both RSV and AGK-2 caused cytotoxic effects.

As a consequence of exogenous insults, glial cells lost their physiological functions and acquire a reactive phenotype, characterized by profound morphological and functional alterations, such GFAP and S100B overexpression (O'Callaghan and Sriram, 2005; Donato et al., 2013). In our model, we detected marked alteration of both these proteins. In fact, Western blot analysis showed that astrocytes express higher GFAP and S100B protein levels after Aβ challenge. Interestingly, RSV and AGK-2 negatively modulated the expression of both GFAP and S100B.

As mentioned before, the direct correlation between the Aβ-induced toxicity and the production of pro-inflammatory mediators prompted us to investigate the expression of the two main inducible enzymes related to inflammation, iNOS and COX-2. In our experimental condition, we highlighted the existence of an inflammatory state induced by Aβ 1-42 treatment, as detected by the increased expression of both iNOS and COX-2. The alteration of these two proteins was significantly blunted by RSV and AGK-2, indicating a key role in regulating astrogliosis and important astrocyte changes, which contribute to disease progression. In the current study it was observed that SIRT1 and SIRT2 can represent promising targets, whose manipulation could prevent

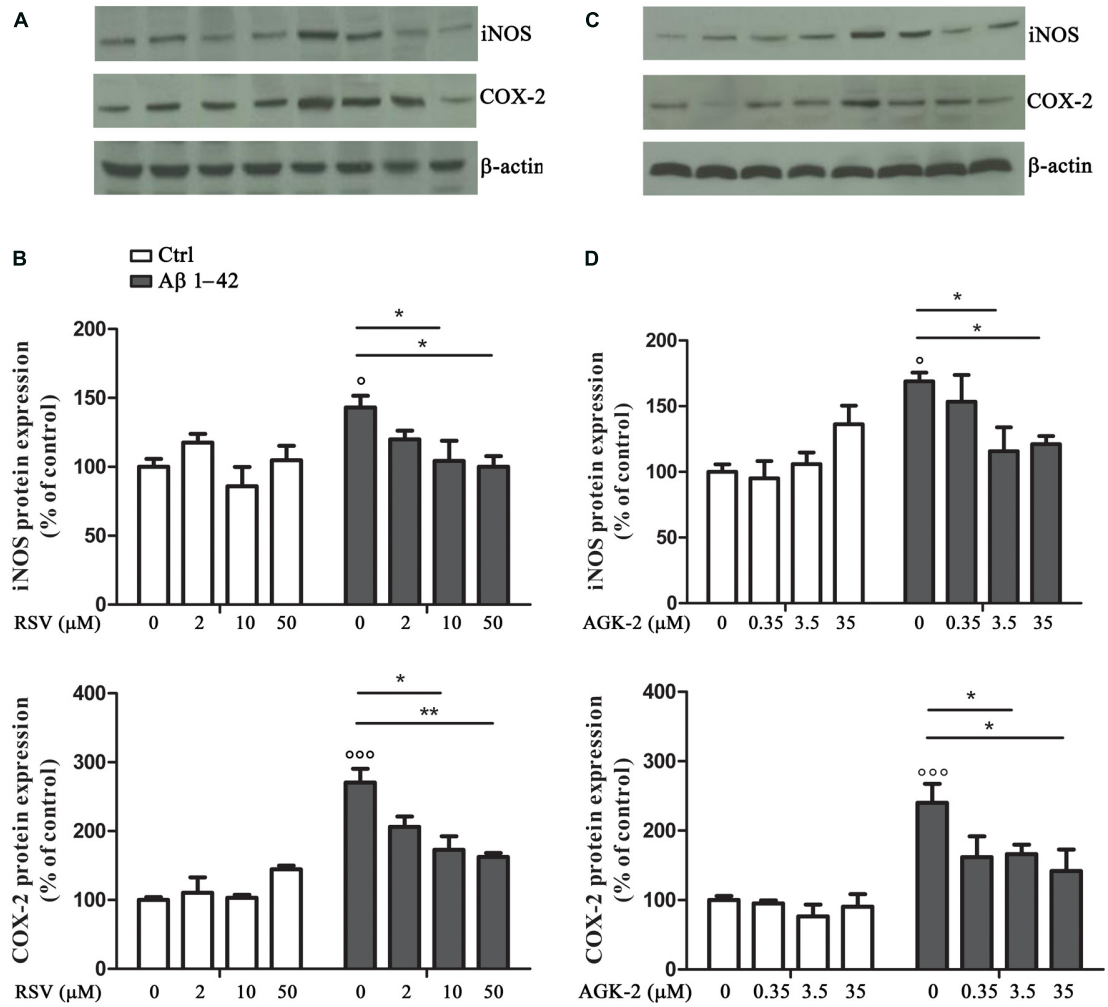

FIGURE 3 | Effect of RSV and AGK-2 on iNOS and COX-2 expression. Astrocytes were treated with Aβ 1-42 (0.23 μM) in the presence of RSV (2 – 10 – 50 μM) or AGK-2 (0.35 – 3.5 – 35 μM). Western blot experiments were carried out 24 h after treatments. Representative immunoblots for iNOS and COX-2 proteins (**A,C** for RSV and AGK-2, respectively). Densitometric analyzes normalized to β-actin loading controls (**B,D** for RSV and AGK-2, respectively). Results are the mean ± SEM of four experiments in triplicate. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison test. $p < 0.001$ and $p < 0.05$ Aβ-challenged versus unchallenged cells; $^{*}p < 0.05$; $^{**}p < 0.01$; for multiple comparison among groups.

over-activation of neuroglia upon pro-inflammatory stimulation. These data suggest a SIRT-dependent mechanism to restrain detrimental effects of excessive astrocyte activation. Moreover, the findings bear major implications in the context of several inflammatory conditions of the central nervous system where astroglia are known to mediate deleterious consequences. In conclusion, the results of the present study provide evidence that SIRT modulation can represent a strategy to counteract reactive gliosis, and suggest new avenues to walk for the discovery of novel and promising therapy for AD.

AUTHOR CONTRIBUTIONS

Caterina Scuderi, Claudia Stecca, Bronzuoli M. Rosanna, Dante Rotili, Sergio Valente, Antonello Mai, Luca Steardo contributed to the work design, the acquisition and interpretation of data. Caterina Scuderi, Claudia Stecca, Bronzuoli M. Rosanna, Dante Rotili, Sergio Valente drafted the manuscript and revised it. Antonello Mai and Luca Steardo approved the final version of the manuscript. Caterina Scuderi, Claudia Stecca, Bronzuoli M. Rosanna, Dante Rotili, Sergio Valente, Antonello Mai, Luca Steardo ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REFERENCES

Allaman, I., Gavillet, M., Bélanger, M., Laroche, T., Viertl, D., Lashuel, H. A., et al. (2010). Amyloid-beta aggregates cause alterations of astrocytic metabolic

phenotype: impact on neuronal viability. *J. Neurosci.* 30, 3326–3338. doi: 10.1523/JNEUROSCI.5098-09.2010

Alzheimer's Association Report. (2013). 2013 Alzheimer's disease facts and figures. *Alzheimers and Dement.* 9, 208–245. doi: 10.1016/j.jalz.2013.02.003

Anekonda, T. S. (2006). Resveratrol–a boon for treating Alzheimer's disease? *Brain Res. Rev.* 52, 316–326. doi: 10.1016/j.brainresrev.2006.04.004

Block, M. L., and Hong, J. S. (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog. Neurobiol.* 76, 77–98. doi: 10.1016/j.pneurobio.2005.06.004

Caricasole, A., Copani. A., Caruso, A., Caraci, F., Iacovelli, L., Sortino, M. A., et al. (2003). The Wnt pathway, cell-cycle activation and beta-amyloid: novel therapeutic strategies in Alzheimer's disease? *Trends Pharmacol. Sci.* 24, 233–238. doi: 10.1016/S0165-6147(03)00100-7

Chen, J., Zhou, Y., Mueller-Steiner, S., Chen, L. F., Kwon, H., Yi, S., et al. (2005). SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. *J. Biol. Chem.* 280, 40364–40374. doi: 10.1074/jbc.M509329200

Craft, J. M., Watterson, D. M., and Van Eldik, L. J. (2006). Human amyloid beta-induced neuroinflammation is an early event in neurodegeneration. *Glia* 53, 484–490. doi: 10.1002/glia.20306

de Oliveira, R. M., Pais, T. F., and Outeiro, T. F. (2010). Sirtuins: common targets in aging and in neurodegeneration. *Curr. Drug Targets* 11, 1270–1280. doi: 10.2174/1389450111007011270

Di Filippo, M., Sarchielli, P., Picconi, B., and Calabresi, P. (2008). Neuroinflammation and synaptic plasticity: theoretical basis for a novel, immune-centred, therapeutic approach to neurological disorders. *Trends Pharmacol. Sci.* 29, 402–412. doi: 10.1016/j.tips.2008.06.005

Donato, R., and Heizmann, C. W. (2010). S100B protein in the nervous system and cardiovascular apparatus in normal and pathological conditions. *Cardiovasc. Psychiatry Neurol.* 2010, 929712. doi: 10.1155/2010/929712

Donato, R., Cannon, B. R., Sorci, G., Riuzzi, F., Hsu, K., Weber, D. J., et al. (2013). Functions of S100 proteins. *Curr. Mol. Med.* 13, 24–57. doi: 10.2174/156652413804486214

Ferreira, S. T., Clarke, J. R., Bomfim, T. R., and De Felice, F. G. (2014). Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease. *Alzheimers Dement.* 10, S76–S83. doi: 10.1016/j.jalz.2013.12.010

Gan, L., and Mucke, L. (2008). Paths of convergence: sirtuins in aging and neurodegeneration. *Neuron* 58, 10–14. doi: 10.1016/j.neuron.2008.03.015

Griffin, W. S. (2006). Inflammation and neurodegenerative diseases. *Am. J. Clin. Nutr.* 83, 470S–474S. doi: 10.1111/j.1365-2567.2009.03225.x

Haigis, M. C., and Sinclair, D. A. (2010). Mammalian sirtuins: biological insights and disease relevance. *Annu. Rev. Pathol.* 5, 253–295. doi: 10.1146/annurev.pathol.4.110807.092250

Harting, K., and Knöll, B. (2010). SIRT2-mediated protein deacetylation: an emerging key regulator in brain physiology and pathology. *Eur. J. Cell Biol.* 89, 262–269. doi: 10.1016/j.ejcb.2009.11.006

Hoppe, J. B., Rattray, M., Tu, H., Salbego, C. G., and Cimarosti, H. (2013). SUMO-1 conjugation blocks beta-amyloid-induced astrocyte reactivity. *Neurosci. Lett.* 546, 51–56. doi: 10.1016/j.neulet.2013.04.050

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., et al. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425, 191–196. doi: 10.1038/nature01960

Jefremov, V., Zilmer, M., Zilmer, K., Bogdanovic, N., and Karelson, E. (2007). Antioxidative effects of plant polyphenols: from protection of G protein signaling to prevention of age-related pathologies. *Ann. N. Y. Acad. Sci.* 1095, 449–457. doi: 10.1196/annals.1397.048

Julien, C., Tremblay, C., Emond, V., Lebbadi, M., Salem, N. Jr., Bennett, D. A., et al. (2009). Sirtuin 1 reduction parallels the accumulation of tau in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 68, 48–58. doi: 10.1097/NEN.0b013e3181922348

Kim, D., Nguyen, M. D., Dobbin, M. M., Fischer, A., Sananbenesi, F., Rodgers, J. T., et al. (2007). SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *EMBO J.* 26, 3169–3179. doi: 10.1038/sj.emboj.7601758

Li, Y., Yokota, T., Gama, V., Yoshida, T., Gomez, J. A., Ishikawa, K., et al. (2007). Bax-inhibiting peptide protects cells from polyglutamine toxicity caused by Ku70 acetylation. *Cell Death Differ.* 14, 2058–2067. doi: 10.1038/sj.cdd.4402219

Lu, T., Pan, Y., Kao, S. Y., Li, C., Kohane, I., Chan, J., et al. (2004). Gene regulation and DNA damage in the ageing human brain. *Nature* 429, 883–891. doi: 10.1038/nature02661

Luthi-Carter, R., Taylor, D. M., Pallos, J., Lambert, E., Amore, A., Parker, A., et al. (2010). SIRT2 inhibition achieves neuroprotection by decreasing sterol biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7927–7932. doi: 10.1073/pnas.1002924107

Meda, L., Baron, P., and Scarlato, G. (2001). Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins. *Neurobiol. Aging* 22, 885–893. doi: 10.1016/S0197-4580(01)00307-4

Menard, C., Bastianetto, S., and Quirion, R. (2013). Neuroprotective effects of resveratrol and epigallocatechin gallate polyphenols are mediated by the activation of protein kinase C gamma. *Front. Cell. Neurosci.* 7:281. doi: 10.3389/fncel.2013.00281

Michan, S., and Sinclair, D. (2007). Sirtuins in mammals: insights into their biological function. *Biochem. J.* 404, 1–13. doi: 10.1042/BJ20070140

Mrak, R. E., and Griffin, W. S. (2001). The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease. *Neurobiol. Aging* 22, 915–922. doi: 10.1016/S0197-4580(01)00293-7

O'Callaghan, J. P., and Sriram, K. (2005). Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert Opin. Drug Saf.* 4, 433–442. doi: 10.1517/14740338.4.3.433

Olabarria, M., Noristani, H. N., Verkhratsky, A., and Rodríguez, J. J. (2010). Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia* 58, 831–838. doi: 10.1002/glia.20967

Outeiro, T. F., Kontopoulos, E., Altmann, S. M., Kufarev, I., Strathearn, K. E., Amore, A. M., et al. (2007). Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* 317, 516–519. doi: 10.1126/science.1143780

Outeiro, T. F., Marques, O., and Kazantsev, A. (2008). Therapeutic role of sirtuins in neurodegenerative disease. *Biochim. Biophys. Acta* 1782, 363–369. doi: 10.1016/j.bbadis. 2008.02.010

Qin, W., Chachich, M., Lane, M., Roth, G., Bryant, M., de Cabo, R., et al. (2006a). Calorie restriction attenuates Alzheimer's disease type brain amyloidosis in Squirrel monkeys (*Saimiri sciureus*). *J. Alzheimers Dis.* 10, 417–422.

Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., et al. (2006b). Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *J. Biol. Chem.* 281, 21745–21754. doi: 10.1074/jbc.M602909200

Repetto, G., del Peso, A., and Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* 3, 1125–1131. doi: 10.1038/nprot.2008.75

Scuderi, C., Esposito, G., Blasio, A., Valenza, M., Arietti, P., Steardo, L. Jr., et al. (2011). Palmitoylethanolamide counteracts reactive astrogliosis induced by β-amyloid peptide. *J. Cell. Mol. Med.* 15, 2664–2674. doi: 10.1111/j.1582-4934.2011.01267.x

Scuderi, C., and Steardo, L. (2013). Neuroglial roots of neurodegenerative diseases: therapeutic potential of palmitoylethanolamide in models of Alzheimer's disease. *CNS Neurol. Disord. Drug Targets* 12, 62–69. doi: 10.2174/18715273113120100011

Scuderi, C., Stecca, C., Iacomino, A., and Steardo, L. (2013). Role of astrocytes in major neurological disorders: the evidence and implications. *IUBMB Life* 65, 957–961. doi: 10.1002/iub.1223

Scuderi, C., Valenza, M., Stecca, C., Esposito, G., Carratù, M. R., and Steardo, L. (2012). Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices via peroxisome proliferator-activated receptor-α. *J. Neuroinflammation* 9, 49. doi: 10.1186/1742-2094-9–21

Spires-Jones, T. L., Fox, L. M., Rozkalne, A., Pitstick, R., Carlson, G. A., and Kazantsev, A. G. (2012). Inhibition of sirtuin 2 with sulfobenzoic acid derivative AK1 is non-toxic and potentially neuroprotective in a mouse model of frontotemporal dementia. *Front. Pharmacol.* 3:42. doi: 10.3389/fphar.2012.00042

Sun, A. Y., Wang, Q., Simonyi, A., and Sun, G. Y. (2010). Resveratrol as a therapeutic agent for neurodegenerative diseases. *Mol. Neurobiol.* 41, 375–383. doi: 10.1007/s12035-010-8111-y

Tang, B. L., and Chua, C. E. (2008). SIRT1 and neuronal diseases. *Mol. Aspects Med.* 29, 187–200. doi: 10.1016/j.mam.2007.02.001

Tuppo, E. E., and Arias, H. R. (2005). The role of inflammation in Alzheimer's disease. *Int. J. Biochem. Cell Biol.* 37, 289–305. doi: 10.1016/j.biocel.2004.07.009

Vairano, M., Dello Russo, C., Pozzoli, G., Battaglia, A., Scambia, G., Tringali, G., et al. (2002). Erythropoietin exerts anti-apoptotic effects on rat microglial cells *in vitro*. *Eur. J. Neurosci.* 16, 584–592. doi: 10.1046/j.1460-9568.2002.02125.x

Verkhratsky, A., and Butt, A. M. (2007). *Glial Neurobiology*. Chichester: John Wiley and Sons Ltd. doi: 10.1002/9780470517796

Wyss-Coray, T., and Rogers, J. (2012). Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature. *Cold Spring Harb. Perspect. Med.* 2:a006346. doi: 10.1101/cshperspect.a006346

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Cellular stress response, redox status, and vitagenes in glaucoma: a systemic oxidant disorder linked to Alzheimer's disease

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Amyloid deposits, constituted of amyloid beta (Aβ) aggregates, are a characteristic feature of several neurodegenerative diseases, such as Alzheimer's, mild cognitive impairment and Parkinson's disease. They also have been recently implicated in the pathogenesis of retinal damage, as well as age-related macular degeneration and glaucoma. Glaucoma is a progressive optic neuropathy characterized by gradual degeneration of neuronal tissue due to retinal ganglion cell loss, associated to visual field loss over time resulting in irreversible blindness. Accumulation of Aβ characterizes glaucoma as a protein misfolding disease, suggesting a pathogenic role for oxidative stress in the pathogenesis of retinal degenerative damage associated to glaucoma. There is a growing body of evidence demonstrating a link between Alzheimer's disease and glaucoma. Further, several heat shock proteins (HSPs) members have been implicated both in neurodegenerative diseases and glaucomatous apoptosis. To maintain redox homeostasis vitagenes, as integrated mechanisms, operate actively to preserve cell survival under condition of stress. Vitagenes encode for sirtuin, thioredoxin and HSPs. The present study was designed to investigate cellular stress response mechanisms in the blood of patients with glaucoma, compared to control subjects. Levels of vitagenes HSP-72, heme oxygenase-1, as well as F2-isoprostanes were significantly higher in the blood of patients with glaucoma than in controls. Furthermore, in the same experimental group increased expression of Trx and sirtuin 1 were measured. Our results sustain the importance of redox homeostasis disruption in the pathogenesis of glaucoma and highlights the opportunity that new therapies that prevents neurodegeneration through non-immunomodulatory mechanisms might be synergistically associated with current glaucoma therapies, thus unraveling important targets for novel cytoprotective strategies.

Keywords: free radicals, stress response, vitagenes, hormesis, antioxidants

INTRODUCTION

Glaucoma is a progressive optic neuropathy characterized by degeneration of neuronal tissue due to loss of retinal ganglion cells (RGCs), with accompanying compromission of visual field over time (Gupta et al., 2006; Quigley and Broman, 2006). It is a leading cause of irreversible blindness estimated to affect 79.6 million people worldwide by 2020 (Hinton et al., 1986; Yoneda et al., 2005). Research studies have demonstrated that RGC damage in glaucoma is not limited to the primary insulted neurons, but also involves neighboring neurons. The increase in the prevalence of glaucoma with age is not accounted for only by the increase in ocular hypertension alone, being accompanied by an increase in the vulnerability of the optic nerve to the effects of glaucoma risk factors which increase as function of age. In particular, factors such as tissue hypoxia, disturbed protein metabolism and oxidative stress have been identified to interact in a vicious cycle underlying the pathogenesis of glaucoma (Calandrella et al., 2007; Gupta et al., 2008), ultimately leading to apoptotic retina ganglion cell death (Tatton et al., 2001; Soti et al., 2005; De la Monte and Wands, 2006). In view of these considerations glaucoma can be viewed as a neurodegenerative disease which, similarly to other neurodegenerative pathologies, i.e., Alzheimer's and Parkinson's disease, where irreversible functional deficit ensues as consequence of neuronal dysfunction and death. There is now a growing body of evidence demonstrating a link between AD and glaucoma.

Amyloid deposits, consisting of Aβ, which are a characteristic feature of several neurodegenerative diseases such as Alzheimer's (AD), mild cognitive impairment, and Parkinson's disease (Hinton et al., 1986) have been recently implicated in the pathogenesis of retinal damage, macular degeneration, and glaucoma (Yoneda et al., 2005). Accordingly, drugs designated to target

β-amyloid (Aβ) has been found to reduce apoptotic degeneration of RGCs, as observed *in vitro* and *in vivo*. Furthermore, the presence of increased levels of Aβ characterizes glaucoma as a protein misfolding disease, also suggesting a role for oxidative stress in the pathogenesis of retinal degenerative damage associated to glaucome. Although oxidative stress has been recognized to play a critical role in the development and progression of glaucoma, yet, the exact mechanisms remain elusive. Oxidative stress can cause oxidative attack to DNA, proteins, and lipids, leading to DNA and protein modification, thus sustaining the pathophysiology of degenerative damage of RGCs (Gupta et al., 2006). Relevant to protein misfolding, of emerging interest are heat shock proteins (HSPs), specialized molecular chaperones which mediate various cellular functions. HSPs are up regulated in response to conditions of stress in order to restore normal cell integrity (Soti et al., 2005). The heat shock response, an important component of vitagene family, contributes to establishing a cytoprotective state in a wide variety of human diseases. Vitagenes include, besides HSPs 70 and 32, the latter also called heme oxygenase-1 (HO-1), thioredoxin and sirtuins (Butterfield et al., 2010, 2011). Several families of HSP have been implicated in neurodegenerative diseases and glaucomatous RGC apoptosis with increased levels of circulating autoantibodies to alpha-crystallins and HSP-27 and increased immunostaining of HSP-60, HSP-27 in RGCs and the retinal blood vessels in glaucoma patients (Shieds et al., 1996; Izzotti et al., 2006). In a rat glaucoma model, treatment with geranylgeranylacetone increases HSP-72 synthesis while reducing markedly RGC loss, possibly through interactions with different protein kinases, such as Akt kinase, and the inhibition of NF-kB. In this study we tested the hypothesis that there may be a causal relationship between AD and glaucoma that may be explained by systemic oxidative stress and dysregulation of cellular stress response. Present work elucidated cellular stress response in peripheral cells in patients with glaucoma as compared to healthy volunteers, as control, in order to gain insight into the pathogenic mechanisms operating in the neurodegenerative damage associated with this disease and exploit the possible role of vitagenes in opening up new therapeutic targets for limiting the oxidative damage associated to degeneration occurring in glaucoma.

MATERIALS AND METHODS

PATIENTS

Eighteen patients (12 males and six females, mean age 60 ± 15 years) with diagnosis of hypertensive primary open-angle glaucoma (POAG), with typical optic nerve head and visual field damage, were included in the study. Mean MD and PSD were respectively −7.5 ± 8.6 dB, and 4.2 ± 3.8 dB. Twenty age-matched healthy volunteers were recruited as controls. Patients and control subjects underwent IOP measurement by Goldmann applanation tonometer, optic nerve head examination by 78 D lens at the slit lamp, and visual field test 24-2 SITA standard, by a 750 Humprey perimeter (HFA II). Clinical characteristics of patients and control subjects are shown in **Table 1**. Patients with normal tension glaucoma, previous uveitis, diabetes, arterial hypertension were excluded. The study was conducted according to guidelines of local Ethics Committee, and informed consent was obtained from all patients.

SAMPLING AND LYMPHOCYTE PURIFICATION

Blood (5 ml) collected from controls and patients into tubes containing EDTA, was divided into two aliquots, 1 and 4 ml respectively. One aliquots (1 ml) was centrifuged at 3000 × *g* for 10 min at 4°C to separate serum from red blood cells, while 4 ml aliquots, were utilized for lymphocytes purification, which was accomplished by using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA).

WESTERN BLOT ANALYSIS

HSP-70, HO-1, Trx, and Sirt-1 protein levels were estimated by Western blot analysis which was accomplished as previously described in Calabrese et al. (2012). Plasma samples were processed as such, while the isolated lymphocyte pellet was homogenized and centrifuged at 10,000 × *g* for 10 min. The supernatant was then used for analysis after determination of protein content. Proteins extracted for each sample, at equal concentration (40 μg), were boiled for 3 min in sample buffer (containing 40 mM Tris-HCl pH 7.4, 2.5% SDS, 5% 2-mercaptoethanol, 5% glycerol, 0.025 mg/ml of bromophenol blue) and then separated on a polyacrylamide mini gels precasting 4–20% (cod NB10420 NuSept Ltd Australia). Separated proteins were transferred onto nitrocellulose membrane (BIO-RAD, Hercules, CA, USA) in transfer buffer containing (0.05% di SDS, 25 mM di Tris, 192 mM glycine and 20% v/v methanol). The transfer of the proteins on the nitrocellulose membrane was confirmed by staining with Ponceau Red which was then removed by three washes in PBS (phosphate buffered saline) for 5 min each. Membranes were then incubated for 1 h at room temperature in 20 mM Tris pH 7.4, 150 mM NaCl and Tween 20 (TBS-T) containing 2% milk powder and incubated with appropriate primary antibodies, namely anti-HSP-70, anti-HO-1, anti-Trx and anti Sirt-1 polyclonal antibody (Santa Cruz Biotech. Inc.), overnight at 4°C in TBS-T. The same membrane was incubated with a goat polyclonal antibody anti-beta-actin (SC 1615 Santa Cruz Biotech. Inc., Santa Cruz, CA, USA, dilution 1:1000) to verify that the concentration of protein loaded in the gel was the same in each sample. Excess unbound antibodies were removed by three washes are with TBS-T for 5 min. After incubation with primary antibody, the membranes were washed three times for 5 min. in TBS-T and then incubated for 1 h at room temperature with the secondary polyclonal antibody conjugated with horseradish peroxidase (dilution 1:500). The membranes were then washed three times with TBS-T for 5 min. Finally, the membranes were incubated for 3 min with SuperSignal chemiluminiscence detection system kit (Cod 34080 Pierce Chemical Co, Rockford, IL, USA) to display the specific protein bands for each antibody. The immunoreactive bands were quantified by capturing the luminescence signal emitted from the membranes with the Gel Logic 2200 PRO (Bioscience) and analyzed with Molecular Imaging software for the complete analysis of regions of interest for measuring expression ratios. The molecular weight of proteins analyzed was determined using a standard curve prepared with protein molecular weight.

Table 1 | Clinical data of glaucoma patients and control subjects.

	Number of subjects	Age (Mean ± SD)	Gender (F/M)	md (Mean ± SD)	Psd (Mean ± SD)
Patients	18	60 ± 15	7/1	−7.5 ± 8.6	4.2 ± 3.8
Controls	20	73 ± 5	2/8	−1.2 ± 1.1	0.8 ± 0.3

Md, mean defect; psd, pattern standard deviation.

MEASUREMENT OF F2-ISOPROSTANES

F2-isoprostanes were determined by HPLC according to the procedure of Ritov et al. (2002). F2-isoprostane content in plasma was expressed in nM.

DETERMINATION OF PROTEIN

Protein concentration in all experimental samples was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) according to Smith et al. (1985) and using serum bovine albumin as standard.

STATISTICAL ANALYSIS

Results were expressed as means ± S.E.M. Each experiment was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $P < 0.05$.

RESULTS

In our study we evaluated the expression of stress proteins in plasma and lymphocytes of glaucomatous patients compared to controls. Among the 70 kDa family of HSPs we evaluated the inducible HSP-70 (HSP-72) isoform and its expression. As shown in **Figure 1A**, a significant ($P < 0.05$) increase in HSP-70 was found in lymphocytes of patients with glaucoma with respect to healthy control subjects. A representative immunoblot is reported in **Figure 1B**. Western blot analysis of plasma probed for HSP-70 are reported in **Figure 2A**, which shows that HSP-70 expression increased significantly ($P < 0.05$) in patients with glaucoma, compared to controls. A representative immunoblot is shown in **Figure 2B**.

Heme oxygenase-1, another HSP-32 endowed with cytoprotective properties (Soti et al., 2005), was found expressed at significantly higher levels in lymphocytes of patients with glaucoma than in controls (**Figure 3A**). A representative immunoblot is illustrated in **Figure 3B**. Similarly to lymphocyte finding, patients with glaucoma exhibited higher level plasma HO-1 protein then healthy controls (**Figures 4A,B**).

Analysis of lymphocytes in patients with glaucoma, compared to control group, revealed a significant ($P < 0.05$) increase of Trx expression (**Figure 5A**), while in the plasma there was no statistically significant difference between the two experimental groups (**Figure 6A**). Representative immunoblots are reported in **Figures 5B** and **6B**, respectively.

Interestingly, we found significantly ($P < 0.05$) higher levels of sirtuin-1 in lymphocytes of patients with glaucoma than in the control group (**Figure 7A**). Consistent with the changes found in lymphocytes, analysis of plasma confirmed increased protein levels of sirtuin-1, higher in patients with glaucoma as to compare with the healthy control group (**Figure 8A**). Representative immunoblots are shown in **Figures 7B** and **8B**, respectively. As to our knowledge this is the first evidence of changes in sirtuin-1 expression in glucomatous pathology, although this finding may not be a marker specific for this progressive chronic inflammatory systemic disease.

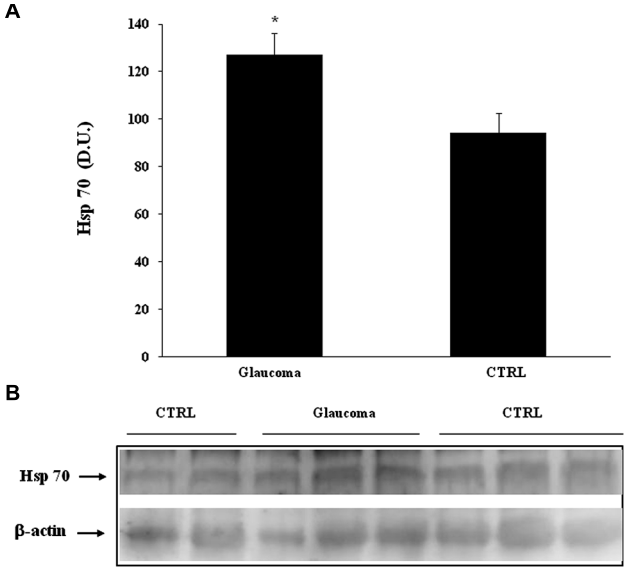

FIGURE 1 | (A) HSP-70 protein levels in plasma of glaucoma and control subjects. Samples from control and glaucoma patients were assayed for HSP-70 expression by Western blot. A representativ eimmunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. *$P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

Further, we evaluated systemic pro-oxidant conditions, by measuring lipid-derived circulating F2 isoprostanes. We found a significant increase ($P < 0.05$) of total F2-isoprostanes in the plasma of patients with glaucoma ($P < 0.05$) with respect to controls (**Figure 9**).

DISCUSSION

Glaucoma is one of the leading causes of vision loss worldwide. Open-angle glaucoma, the most common form of glaucoma, is characterized by a progressive loss of RGCs and atrophy of the optic nerve, resulting in loss of visual field (Hinton et al., 1986; Yoneda et al., 2005). Several theories have been proposed, including mechanical and vascular pathogenesis for the

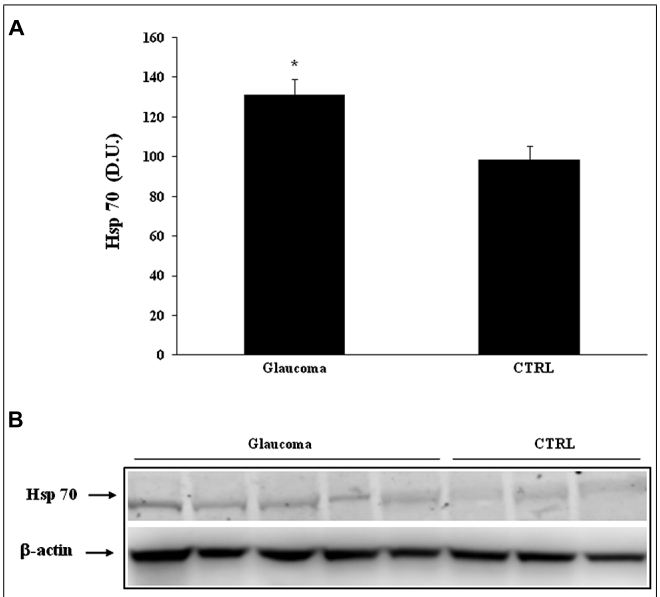

FIGURE 2 | (A) HSP-70 protein levels in lymphocytes of glaucoma and control subjects. Samples from controls and glaucoma patients were assayed for HSP-70 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

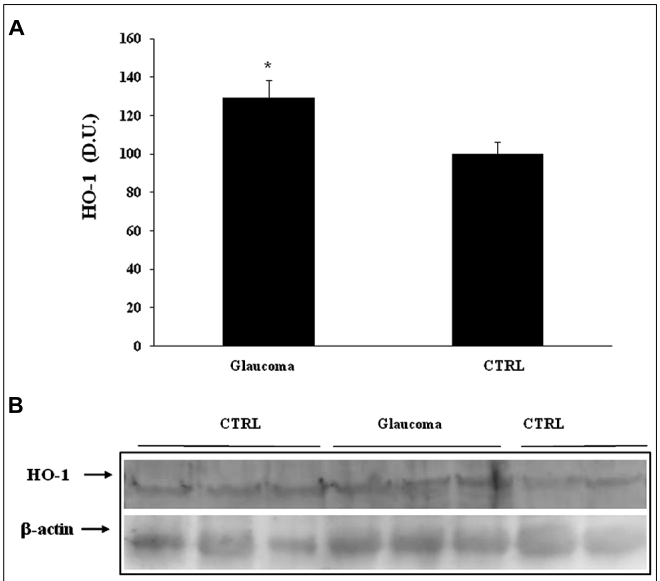

FIGURE 3 | (A) HO-1 protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for HO-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

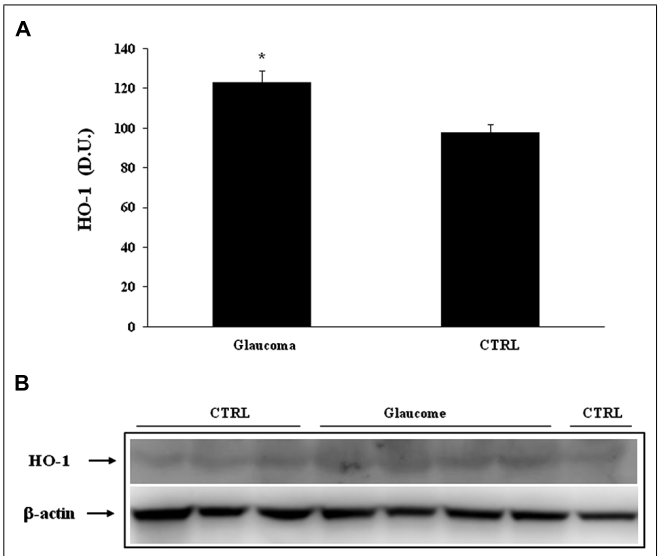

FIGURE 4 | (A) HO-1 protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for HO-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

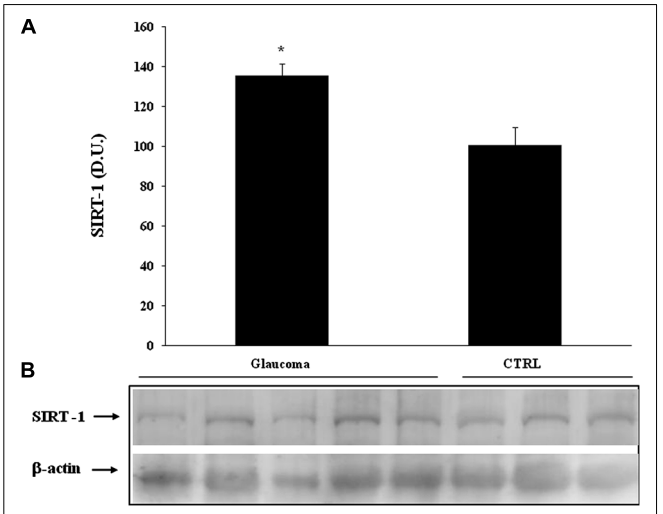

FIGURE 5 | (A) Sirtuin-1 protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Sirt-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of three independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

glaucomatous optic neuropathy. Elevated intraocular pressure is a strong risk factor, but a subset of glaucoma patients has normal intraocular pressure designating a normal tension glaucoma. Clearly, other factors different from intraocular pressure, such as genetic factors, are thought to be involved in RGC apoptotic cell death in glaucoma. The e4 allele of the APOE gene has been

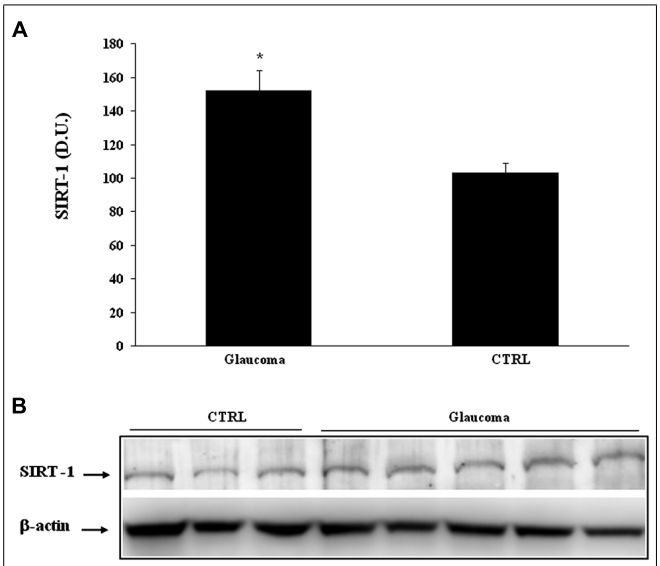

FIGURE 6 | (A) Sirtuin-1 protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Sirt-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

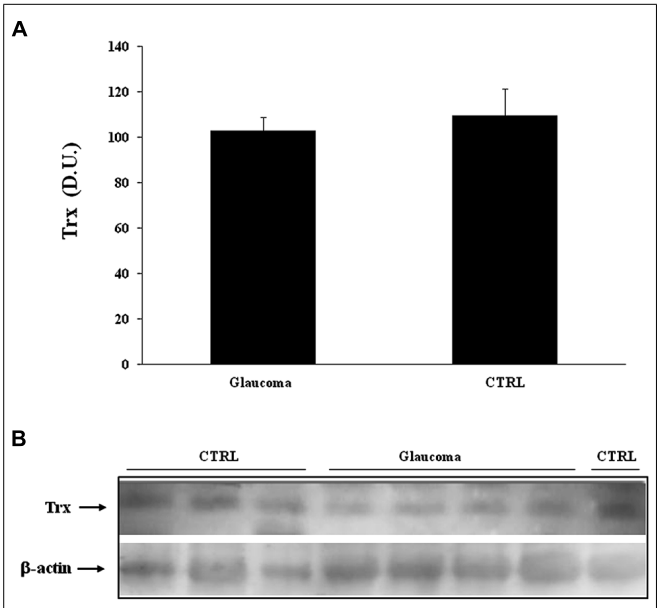

FIGURE 7 | (A) Thioredoxin protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Trx expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

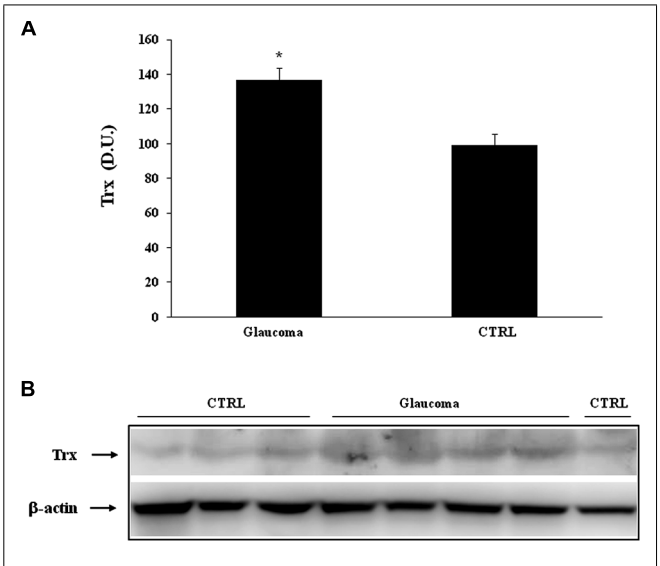

FIGURE 8 | (A) Thioredoxin protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Trx expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. **(B)** Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $*P \leq 0.05$ vs. control. D.U., densitometric units; CTRL, control.

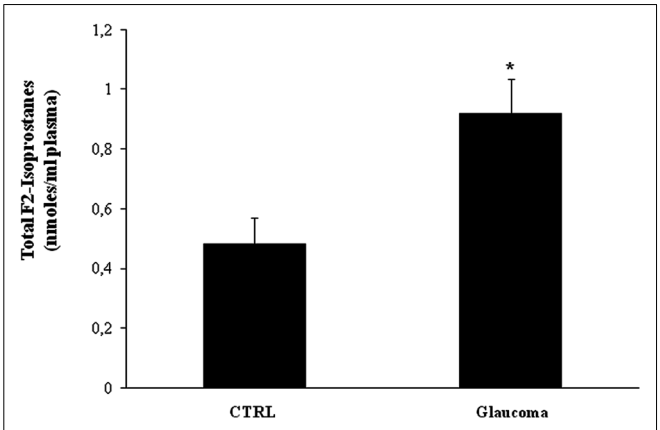

FIGURE 9 | Total F2-isoprostanes levels in plasma glaucoma patients. Plasma samples from patients with glaucoma and age-matched controls were assayed for total F2-isoprostanes. Data are expressed as mean ± SEM of 18 to 20 patients per group. $*P < 0.05$ vs. controls.

also considered in the pathophysiology of open-angle glaucoma, although the question still remains elusive (Quigley and Broman, 2006).

Oxidative stress is considered an important risk factor for the development of primary angle-closure glaucoma and increased levels of oxidative stress products have been documented in primary angle-closure glaucoma (Shieds et al., 1996; Izzotti et al., 2006; Butterfield et al., 2011). Visual loss which often starts in the periphery and advances involving central vision, has devastating consequences to patient's quality of life (Gupta et al., 2006; Quigley and Broman, 2006).

We have recently demonstrated that increased oxidative stress and cellular stress response are a systemic presentation of the oxidative burden occurring in AD patients, rising the conceivable possibility that Alzheimer's disease might not be exclusively a primary neurological pathology rather being a systemic oxidant disorder (Siciliano et al., 2011; Cornelius et al., 2013). In this study we hypothesize that there may be a causal relationship between AD and glaucoma that may be explained by systemic oxidative stress and dysregulation of cellular stress response. We have found in patients with glaucoma a systemic condition of oxidative stress as revealed by upregulation of lipid-derived F2 isoprostanes. This marker of oxidative stress was found in the blood of patients with glaucoma at significantly higher levels than in controls. Similarly to other oxidant disorders, such as AD (Calabrese et al., 2012; Mayeux and Stern, 2012) or multiple sclerosis (Calabrese et al., 2010b) a direct relationship, although not necessarily causal, may exist between organ specific pathology and systemic alterations underlying or reflecting the local oxidative status (Ferreira et al., 2004). Reactive oxygen species (ROS) are an essential component of intracellular signaling network, regulated through the intrinsic antioxidant capacity of a cell, but when ROS formation exceedingly increases damage to DNA, proteins, and lipids macromolecules ensues. During cellular metabolism mitochondrial compartment accounts for the major source of ROS generation. However, excess in free radical production induces oxidative stress and damage. Growing evidence now sustains a critical role for free radical-induced oxidative damage in glaucomatous neurodegeneration occurring in different subcellular compartments of RGCs. Consistent with this notion, oxidatively modified proteins and advanced glycation end products accumulate in glaucomatous neurodegeneration, thus increasing neuronal susceptibility to glial dysfunction (Shields et al., 1996; Sloane et al., 2002; Tamura et al., 2006). This last event, in turn, contributes to propagate neuronal damage resulting in secondary degenerative damage. Furthermore, free radical-mediated oxidative insult in glaucoma enhances antigen presenting activity of glial cells and hence stimulates immune response (Tezel, 2006; Calandrella et al., 2007).

Oxidative damage is one of the most important causes of brain protein damage and dysfunction in several age-related neurodegenerative disorders including Alzheimer's disease (Sloane et al., 2002). RGCs and the optic nerve have demonstrated similar mechanisms of cell death in glaucoma to those of Alzheimer's disease, marking glaucoma as a neurodegenerative disease (Guo et al., 2006; Pennisi et al., 2011). AD is a progressive neurodegenerative disorder characterized by cognitive and memory deterioration, as well as changes in personality, behavioral disturbances and an impaired ability to perform activities of daily living (McKinnon, 2003; Wostyn et al., 2009; Siciliano et al., 2011). AD is known to be the most common form of dementia and is a major public health problem throughout the world (Calabrese et al., 2012). In addition to synaptic degradation and extensive neuronal cell loss, neuropathological characteristics of AD include extracellular senile plaques containing β-amyloid (Aβ) derived from β-amyloid precursor protein (APP) after sequential cleavage by b-secretase and c-secretase, and intracellular neurofibrillary tangles caused by abnormally phosphorylated tau protein (De la Monte and Wands, 2006).

There is a growing body of evidence demonstrating a link between AD and glaucoma. However, the nature of this link remains obscure. Interestingly, recently published research may provide a clue toward a better understanding of the high rate of comorbidity reported between AD and glaucoma.

It is intriguing to note that AD and glaucoma have many common features. Both are slow and chronic neurodegenerative disorders with a strong age-related incidence. Studies consistently report decreased levels of β-amyloid and increased levels of tau in cerebrospinal fluid from AD patients in comparison with healthy subjects. Similarly, decreased levels of β-amyloid and significantly increased levels of tau have been detected in the vitreous fluid from patients with glaucoma or diabetic retinopathy in comparison with the levels in a control group (Calabrese et al., 2010b; Pennisi et al., 2011; Siciliano et al., 2011; Cornelius et al., 2013). This finding corroborates a role for β-amyloid and tau in the pathogenesis of glaucoma, suggesting that the neurodegenerative process in these ocular diseases might share, at least in part, a common mechanism with AD. It was also demonstrated recently that abnormal tau AT8 is present in human glaucomas with uncontrolled elevated intraocular pressure. Furthermore, there is evidence of a build-up of Aβ in RGCs in experimental rat glaucoma. Activation of caspases and abnormal APP processing, which includes production of Aβ are important events in AD (Bullock and Hammond, 2003; Guo et al., 2006).

To gain further insight into the role of oxidant/antioxidant balance in the pathogenesis of glaucoma, in addition to oxidative stress, expression of Sirt-1 and Trx was determined in the peripheral blood of glaucomatous patients. Interestingly, levels of vitagenes HSP-72 and HO-1 were significantly higher in the blood of patients with glaucoma than in controls. These changes were associated with an increased expression of Trx and sirtuin 1 in the same experimental group.

To adapt to environmental changes and survive different types of injuries, as in the case of acute or chronic stress, exposed cells are continually challenged to activate integrated survival responses (Bullock and Hammond, 2003). One of these, the heat shock response actively operate in the optic cell system, under control of redox regulated gene network, the vitagene network, recognized to be critical for the intracellular chaperoning function which is essential for the proper folding of misfolded or mutated proteins, thereby protecting vulnerable cells from death (Selkoe, 2001; Rocchi et al., 2003; Guo et al., 2006). As stress inducible proteins, chaperones help the correct folding and maintenance of the proper conformation of essential proteins, thus promoting cell survival in all those pathological conditions associated oxidative stress (Hirota et al., 2002; Calabrese et al., 2010c). Under oxidative stress conditions, such as that found in patients with glaucoma, HO-1 was also found increased in lymphocytes and plasma of patients with glaucoma. HO-1 is an early gene induced by oxidative stress producing powerful antioxidant and antinitrosative molecules such as biliverdin and bilirubin (Halliwell, 2006; Calabrese et al., 2010a, 2013). HO-1 increase in the lymphocytes of patients with glaucoma may indicate that, in

response to an oxidant insult, induction of an early gene is a significant part of the antioxidant response which might have biological relevance considering the long term course of the disease. Under stress conditions, induction of sirtuins is a well recognized defense mechanism against oxidative injury, representing a common feature in a number of neurodegenerative diseases (Salminen et al., 2008). Here we found that the levels of Sirt-1 in glaucoma lymphocytes were significantly higher than in controls, a finding associated with increased content of F2-isoprostanes as marker of oxidative stress. This is relevant to the pathogenesis of glaucoma. Several studies suggest that the Sirt-1 gene is redox-regulated and its expression appears closely related to conditions of oxidative stress (Drake et al., 2003; Bonda et al., 2011). Thus, its induction could represent a protective system potentially active against brain oxidative injury (Kessing et al., 2007; Herranz and Serrano, 2010). In addition, another protein, thioredoxin (Trx), which is emerging as critical vitagene involved in brain stress tolerance was found increased in the same experimental group (Tanaka et al., 2000; Tonissen and Trapani, 2009). Besides its role in the protection against oxidative stress, Trx is critically involved in the regulation of cell growth and cell death (Yi and Luo, 2010; Di Paola et al., 2011). Consistently, modulation of endogenous cellular defense mechanisms such as the vitagene network, including HSPs, sirtuin, and thioredoxin proteins may open a new approaches to therapeutic interventions in diseases associated with tissue damage and cell death, such as in glaucomatous neurodegeneration (Dali-Youcef et al., 2007; Dumont et al., 2009; Ballard et al., 2011). Our data are in favor of the hypothesis linking oxidative stress to the pathogenesis of glaucoma, and indicate that stress responsive genes may represent an important target for novel cytoprotective strategies, as molecules inducing this defense mechanism, via nutritional and/or pharmacological approaches, can exploit the potential for antidegenerative therapeutic interventions.

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REFERENCES

Ballard, C., Gauthier, S., Corbett, A., Brayne, C., Aarsland, D., and Jones, E. (2011). Alzheimer's disease. *Lancet* 19, 1019–1031. doi: 10.1016/S0140-6736(10)61349-9

Bonda, D. J., Lee, H. G., Camins, A., Pallàs, M., Casadesus, G., Smith, M. A., et al. (2011). The sirtuin pathway in ageing and Alzheimer disease: mechanistic and therapeutic consideration. *Lancet Neurol.* 10, 275–279. doi: 10.1016/S1474-4422(11)70013-8

Bullock, R., and Hammond, G. (2003). Realistic expectations: the management of severe Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* 17, S80–S85. doi: 10.1097/00002093-200307003-00004

Butterfield, D. A., Bader Lange, M. L., and Sultana, R. (2010). Involvements of the lipid peroxidation product, HNE, in the pathogenesis and progression of Alzheimer's disease. *Biochim. Biophys. Acta* 1801, 924–929. doi: 10.1016/j.bbalip.2010.02.005

Butterfield, D. A., Reed, T., and Sultana, R. (2011). Roles of 3-nitrotyrosine- and 4-hydroxynonenalmodified brain proteins in the progression and pathogenesis of Alzheimer's disease. *Free Radic. Res.* 45, 59–72. doi: 10.3109/10715762.2010.520014

Calabrese, E. J., Iavicoli, I., and Calabrese, V. (2013). Hormesis: its impact on medicine and health. *Hum. Exp. Toxicol.* 32, 120–152. doi: 10.1177/0960327112455069

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Calabrese, E. J., and Mattson, M. P. (2010a). Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. *Antioxid. Redox Signal.* 13, 1763–1811. doi: 10.1089/ars.2009.3074

Calabrese, V., Cornelius, C., Giuffrida, A. M., and Calabrese, E. J. (2010b). Cellular stress responses, mitostress and carnitine insufficiencies as critical determinants in aging and neurodegenerative disorders: role of hormesis and vitagenes. *Neurochem. Res.* 35, 1880–1915. doi: 10.1007/s11064-010-0307-z

Calabrese, V., Cornelius, C., Trovato, A., Cavallaro, M., Mancuso, C., Di Rienzo, L., et al. (2010c). The hormetic role of dietary antioxidants in free radical-related diseases. *Curr. Pharm. Des.* 16, 877–883. doi: 10.2174/138161210790883615

Calabrese, V., Cornelius, C., Leso, V., Trovato-Salinaro, A., Ventimiglia, B., Cavallaio, M., et al. (2012). Oxidative stress, glutathione status, sirtuin and cellular stress response in type 2 diabetes. *Biochim. Biophys. Acta* 1822, 729–736. doi: 10.1016/j.bbadis.2011.12.003

Calandrella, N., Scarsella, G., Pescosolido, N., and Risuleo, G. (2007). Degenerative and apoptotic events at retinal and optic nerve level after experimental induction of ocular hypertension. *Mol. Cell. Biochem.* 301, 155–163. doi: 10.1007/s11010-006-9407-0

Cornelius, C., Trovato Salinaro, A., Scuto, M., Fronte, V., Cambria, M. T., Pennisi, M., et al. (2013). Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes. *Immun. Ageing* 10:41. doi: 10.1186/1742-4933-10-41

Dali-Youcef, N., Lagouge, M., Froelich, S., Koehl, C., Schoonjans, K., and Auwerx, J. (2007). Sirtuins: the "magnificent seven," function, metabolism and longevity. *Ann. Med.* 39, 335–345. doi: 10.1080/07853890701408194

De la Monte, S. M., and Wands, J. R. (2006). Molecular indices of oxidative stress and mitochondrial dysfunction occur early and often progress with severity of Alzheimer's disease. *J. Alzheimers Dis.* 9, 167–181.

Di Paola, R., Impellizzeri, D., Trovato Salinaro, A., Mazzon, E., Bellia, F., Cavallaro, M., et al. (2011). Administration of carnosine in the treatment of acute spinal cord injury. *Biochem. Pharmacol.* 82, 1478–1489. doi: 10.1016/j.bcp.2011.07.074

Drake, J., Link, C. D., and Butterfield, D. A. (2003). Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1-42) in a transgenic *Caenorhabditis elegans* model. *Neurobiol. Aging* 24, 415–420. doi: 10.1016/S0197-4580(02)00225-7

Dumont, M., Wille, E., Stack, C., Calingasan, N. Y., Beal, M. F., and Lin, M. T. (2009). Reduction of oxidative stress, amyloid deposition, and memory deficit by manganese superoxide dismutase overexpression in a transgenic mouse model of Alzheimer's disease. *FASEB J.* 23, 2459–2466. doi: 10.1096/fj.09-132928

Ferreira, S. M., Lerner, S. F., Brunzini, R., Evelson, P. A., and Llesuy, S. F. (2004). Oxidative stress markers in aqueous humor of glaucoma patients. *Am. J. Ophthalmol.* 137, 62–69. doi: 10.1016/S0002-9394(03)00788-8

Guo, L., Salt, T. E., Maass, A., Luong, V., Moss, S. E., Fitzke, F. W., et al. (2006). Assessment of neuroprotective effects of glutamate modulation on glaucoma-related retinal ganglion cell apoptosis in vivo. *Invest. Ophthalmol. Vis. Sci.* 47, 626–633. doi: 10.1167/iovs.05-0754

Gupta, N., Ang, L. C., Noel de Tilly, L., Bidaisee, L., and Yucel, Y. H. (2006). Human glaucoma and neural degeneration in intracranial optic nerve, lateral geniculate nucleus, and visual cortex. *Br. J. Ophthalmol.* 90, 674–678. doi: 10.1136/bjo.2005.086769

Gupta, N., Fong, J., Ang, L. C., and Yucel, Y. H. (2008). Retinal tau pathology in human glaucomas. *Can. J. Ophthalmol.* 43, 53–60. doi: 10.3129/i07-185

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* 97, 1634–1658. doi: 10.1111/j.1471-4159.2006.03907.x

Herranz, D., and Serrano, M. (2010). SIRT1: recent lessons from mouse models. *Nat. Rev. Cancer* 10, 819–823. doi: 10.1038/nrc2962

Hinton, D. R., Sadun, A. A., Blanks, J. C., and Miller, C. A. (1986). Optic-nerve degeneration in Alzheimer's disease. *N. Engl. J. Med.* 315, 485–487. doi: 10.1056/NEJM198608213150804

Hirota, K., Nakamura, H., Masutani, H., and Yodoi, J. (2002). Thioredoxin superfamily and thioredoxin-inducing agents. *Ann. N. Y. Acad. Sci.* 957, 189–199. doi: 10.1111/j.1749-6632.2002.tb02916.x

Izzotti, A., Bagnis, A., and Saccà, S. C. (2006). The role of oxidative stress in glaucoma. *Mutat. Res.* 612, 105–114. doi: 10.1016/j.mrrev.2005.11.001

Kessing, L. V., Lopez, A. G., Andersen, P. K., and Kessing, S. V. (2007). No increased risk of developing Alzheimer disease in patients with glaucoma. *J. Glaucoma* 16, 47–51. doi: 10.1097/IJG.0b013e31802b3527

Mayeux, R., and Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 1, 2–8. doi: 10.1101/cshperspect.a006239

McKinnon, S. J. (2003). Glaucoma: ocular Alzheimer's disease? *Front. Biosci.* 8:1140–1156. doi: 10.2741/1172

Pennisi, G., Cornelius, C., Cavallaro, M. M., Trovato Salinaro, A., Cambria, M. T., Pennisi, M., et al. (2011). Redox regulation of cellular stress response in multiple sclerosis. *Biochem. Pharmacol.* 82, 1490–1499. doi: 10.1016/j.bcp.2011.07.092

Quigley, H. A., and Broman, A. T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. *Br. J. Ophthal.* 90, 262–267. doi: 10.1136/bjo.2005.081224

Ritov, V. B., Kelley, D. E., and Kagan, V. E. (2002). Derivatization of F2-isoprostanes with 1-pyrenyldiazomethane and their subsequent determination by fluorescence high-performance liquid chromatography. *Anal. Biochem.* 311, 10–18. doi: 10.1016/S0003-2697(02)00392-5

Rocchi, A., Pellegrini, S., Siciliano, G., and Murri, L. (2003). Causative and susceptibility genes for Alzheimer's disease: a review. *Brain Res. Bull.* 61, 1–24. doi: 10.1016/S0361-9230(03)00067-4

Salminen, A., Kauppinen, A., Suuronen, T., and Kaarniranta, K. (2008). SIRT1 longevity factor suppresses NF-kappaB-driven immune responses: regulation of aging via NF- kappaB acetylation? *Bioessays* 30, 939–942. doi: 10.1002/bies.20799

Selkoe, D. J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* 81, 741–766.

Shields, M. B., Ritch, R., and Krupin, T. (1996). "Classification of the glaucoma," in *The Glaucomas,* 2nd Edn, eds R. Ritch, M. B. Shields, and T. Krupin (St. Louis: Mosby), 717–725.

Siciliano, R., Barone, E., Calabrese, V., Rispoli, V., Butterfield, D. A., and Mancuso, C. (2011). Experimental research on nitric oxide and the therapy of Alzheimer disease: a challenging bridge. *CNS Neurol. Disord. Drug Targets* 10, 766–776. doi: 10.2174/187152711798072356

Sloane, P. D., Zimmerman, S., Suchindran, C., Reed, P., Wang, L., Boustani, M., et al. (2002). The public health impact of Alzheimer's disease 2000-2050: potential implication of treatment advances. *Annu. Rev. Public Health* 23, 213–231. doi: 10.1146/annurev.publhealth.23.100901.140525

Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., et al. (1985). Measurement of proteins using bicinchoninic acid. *Anal. Biochem.* 150, 76–85. doi: 10.1016/0003-2697(85)90442-7

Soti, C., Nagy, E., Giricz, Z., Vigh, L., Csermely, P., and Ferdinandy, P. (2005). Heat shock proteins as emerging therapeutic targets. *Br. J. Pharmacol.* 146, 769–780. doi: 10.1038/sj.bjp.0706396

Tamura, H., Kawakami, H., Kanamoto, T., Kato, T., Yokoyama, T., Sasaki, K., et al. (2006). High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease. *J. Neurol. Sci.* 246, 79–83. doi: 10.1016/j.jns.2006.02.009

Tanaka, T., Nakamura, H., Nishiyama, A., Hosoi, F., Masutani, H., Wada, H., et al. (2000). Redox regulation by thioredoxin superfamily: protection against oxidative stress and aging. *Free Radic. Res.* 33, 851–855. doi: 10.1080/10715760000301361

Tatton, W. G., Chalmers-Redman, R. M., and Tatton, N. A. (2001). Apoptosis and anti-apoptosis signalling in glaucomatous retinopathy. *Eur. J. Ophthalmol.* 11, S12–S22.

Tezel, G. (2006). Oxidative stress in glaucomatous neurodegeneration: mechanisms and consequences. *Prog. Retin. Eye Res.* 25, 490–513. doi: 10.1016/j.preteyeres.2006.07.003

Tonissen, K. F., and Trapani, G. D. (2009). Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. *Mol. Nutr. Food Res.* 53, 87–103. doi: 10.1002/mnfr.200700492

Wostyn, P., Audenaert, K., and De Deyn, P. P. (2009). Alzheimer's disease and glaucoma: is there a causal relationship? *Br. J. Ophthalmol.* 93, 1557–1559. doi: 10.1136/bjo.2008.148064

Yi, J., and Luo, J. (2010). SIRT1 and p53, effect on cancer, senescence and beyond. *Biochim. Biophys. Acta* 1804, 1684–1689. doi: 10.1016/j.bbapap.2010.05.002

Yoneda, S., Hara, H., Hirata, A., Fukushima, M., Inomata, Y., and Tanihara, H. (2005). Vitreous fluid levels of beta-amyloid((1–42)) and tau in patients with retinal diseases. *Jpn. J. Ophthalmol.* 49, 106–108. doi: 10.1007/s10384-004-0156-x

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Osteoporosis and Alzheimer pathology: role of cellular stress response and hormetic redox signaling in aging and bone remodeling

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Alzheimer's disease (AD) and osteoporosis are multifactorial progressive degenerative disorders. Increasing evidence shows that osteoporosis and hip fracture are common complication observed in AD patients, although the mechanisms underlying this association remain poorly understood. Reactive oxygen species (ROS) are emerging as intracellular redox signaling molecules involved in the regulation of bone metabolism, including receptor activator of nuclear factor-κB ligand-dependent osteoclast differentiation, but they also have cytotoxic effects that include lipoperoxidation and oxidative damage to proteins and DNA. ROS generation, which is implicated in the regulation of cellular stress response mechanisms, is an integrated, highly regulated, process under control of redox sensitive genes coding for redox proteins called vitagenes. Vitagenes, encoding for proteins such as heat shock proteins (Hsps) Hsp32, Hsp70, the thioredoxin, and the sirtuin protein, represent a systems controlling a complex network of intracellular signaling pathways relevant to life span and involved in the preservation of cellular homeostasis under stress conditions. Consistently, nutritional anti-oxidants have demonstrated their neuroprotective potential through a hormetic-dependent activation of vitagenes. The biological relevance of dose–response affects those strategies pointing to the optimal dosing to patients in the treatment of numerous diseases. Thus, the heat shock response has become an important hormetic target for novel cytoprotective strategies focusing on the pharmacological development of compounds capable of modulating stress response mechanisms. Here we discuss possible signaling mechanisms involved in the activation of vitagenes which, relevant to bone remodeling and through enhancement of cellular stress resistance provide a rationale to limit the deleterious consequences associated to homeostasis disruption with consequent impact on the aging process.

Keywords: oxidative stress, redox status, Alzheimer's disease, cellular stress response, hormesis, vitagenes

INTRODUCTION

Mitochondrial medicine is emerging as powerful candidate for expanding anatomical and Mendelian biological concepts aimed to solve complexity in age-related diseases, aging and cancer (Di Domenico et al., 2010; Wallace, 2010, 2012, 2013). Interaction between structure and energy is a fundamental life prerequisite. This dualism in eukaryotic cell, was generated approximately two billion years ago after the symbiosis of a glycolytic progenitor, which evolved the nucleus–cytosolic compartment, and an oxidative progenitor evolving toward an ancient mitochondrion. Initially each proto-organism contained all the genes for an independent life. However, 1.2 billion years later, after subsequent genomic reorganizations and alternative rearrangements, was achieved a cellular arrangement in which the mitochondrial compartment became specialized in the generation of energy, while the nuclear and cytosolic compartment was functionally polarized toward structure. This final arrangement was the starting point for multicellularity fostering evolution in higher plants and animal kingdom, including humans. Notably, this original architecture comeback powerfully in our cells in all conditions where tumor initiation and promotion occur and the glycolytic tone of metabolic potential put in motion a process leading to the marginalization of energy transduction mechanisms, kicking off mitochondrial energy production in favor of a sustained high proliferative potential which underlie tumor progression (Calabrese et al., 2007c; Wallace, 2008; Perluigi et al., 2010; Bellia et al., 2011). In view of this, comprehension of aging mechanisms of aging and determinants of life span will contribute to decrease age-related morbidity and promote healthy aging. Over the last centuries, as a consequence of exogenous environmental factors

and medical progress, average lifespan has increased, but maximal life span has not changed (Edrey et al., 2014; Willcox and Willcox, 2014). Consistent with this notion and relevant to longevity mechanisms, vitagenes by preserving cellular homeostasis during stressful conditions represent a functional network regulating life span. Vitagenes, are redox sensitive genes encoding for heat shock proteins (Hsps) Hsp32, Hsp70, for the thioredoxin and the sirtuin protein systems (Calabrese et al., 2008e; Bellia et al., 2009; Cornelius et al., 2013b). In this regard, nutritional antioxidants, have recently been demonstrated to be neuroprotective via hormetic pathways, including vitagenes. The hormetic feature of dose–response appears to quantitatively describe the limits of biological plasticity across phyla as well as at different levels of biological organization (cell, organ, and organism). Thus, pharmaceutical treatments for a wide range of human conditions can be based upon the hormetic biphasic dose response, as discovered within preclinical evaluations and then directly applied to human populations. The biological relevance of hormetic dose–response affects, ultimately, those strategies pointing to the optimal dosing to patients in the treatment of numerous diseases and, within this context, the heat shock response (HSR) can be considered an important hormetic target to design cytoprotective compounds able of inducing stress response mechanisms. Here we discuss signaling mechanisms activating vitagenes, which are relevant to bone remodeling which, through enhancement of stress resistance provide a rational therapeutic approach to limit the deleterious consequences associated to disruption of homeostasis with consequent impact on the aging process (Murshid et al., 2013; Saibil, 2013).

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is characterized by progressive deterioration of cognitive functions and stress (Calabrese et al., 2006d), with biochemical alterations consisting in the accumulation of amyloid-β (Aβ) protein in the form of senile plaques and intracellular neurofibrillary tangles, associated with hyperphosphorylated tau protein and neuronal cell depletion (Liu and Chan, 2014). Prevalence of AD rises exponentially with age, increasing drastically after 65 years. Sporadic AD, affecting more than 15 million people worldwide, is more common than familial AD (Richard and Brayne, 2014). Mild cognitive impairment (MCI) is regarded as a transition state between normal aging and dementia (Swomley et al., 2013). Importantly, almost one half of these individuals evolves to late onset AD (LOAD), accounting for about 60% of the total cases of dementia in USA and Western countries (Swomley et al., 2013). MCI, which can also be due to severe depression, extensive white matter pathology or severe vitamin B12 deficiency, is characterized by memory impairment with or without compromission in other single or multiple cognitive domains, in the absence of fulfillment of the standardized criteria for dementia (Swomley et al., 2013). The pathological hallmarks of AD are amyloid plaques, formed of Aβ peptide derived from the transmembrane amyloid precursor protein (APP; Siciliano et al., 2011), and neurofibrillary protein tangles, composed of hyperphosphorylated tau, in the temporal lobe and some other brain cortical regions associated with death of neuronal cells and synaptic depletion (Hardas et al., 2013). Given the heterogeneity of the etiologic factors underlying the pathophysiology of AD, although integrated approaches have been elaborated to explain its pathogenesis, such as Aβ aggregation, the precise definition of most critical factors determining the clinical onset and progression of the disease remains and elusive and a difficult task (Siciliano et al., 2011). Aβ peptide (Lodi et al., 2006; Wallace, 2013) has been shown to induce protein oxidation in both *in vitro* and *in vivo* studies (Hardas et al., 2013), and increasing evidence supports the role of free radical reactions in the pathogenesis of the disease. Consistent with this notion, it is demonstrated that these peptides form oligomers exerting neurotoxic effects by enhancing reactive oxygen species (ROS) level in the brain (Hardas et al., 2013). More specifically, Aβ oligomers directly generate H_2O_2 through: (i) a cupper-dependent superoxide dismutase-like activity (Fang et al., 2010), (ii) activation of NADPH-oxidase in astrocytes, (iii) modulation of mitochondrial ROS generation via regulation of activity of enzymes such as Aβ-binding alcohol dehydrogenase and α-ketoglutarate dehydrogenase (Borger et al., 2013). Increased proteotoxic and lipoperoxidative brain damage has been documented in subjects with MCI, compared to normal (Chico et al., 2013; Swomley et al., 2013; Cervellati et al., 2014). Thus, oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle characterize AD as a protein misfolding disease, with protein clearance defects through the ubiquitin-proteasome system (Hong et al., 2014; Valasani et al., 2014). There is strong evidence, also, that APP may act as a trophic factor relevant to neurite outgrowth and synaptogenesis, as well as growth and cell proliferation (Abramov et al., 2009; Jiang et al., 2013; Bukanova et al., 2014; Dawkins and Small, 2014; Hughes et al., 2014). However, future research are required to fully clarify mechanisms of APP action (Dawkins and Small, 2014). In addition, identification of normal physiological functions of Aβ is an extremely important emerging issue, as many therapeutic strategies used for the treatment of AD point to prevent Aβ production or to increase Aβ clearance from the brain. Consistently, it cannot be, so far, ruled out that these strategies does not interfere with important physiological functions (Abramov et al., 2009; Kang et al., 2013; Bukanova et al., 2014; Mizoi et al., 2014; Ponnayyan Sulochana et al., 2014).

Among biochemical alterations occurring in AD pathology, deficit in choline esterase and overstimulation of NMDA receptors are the main target for current therapeutic approaches focusing on symptoms rather than more substantial eradication of the disease. Thus, to date there not drugs are available in the market capable of revert the disease, but only to influence the intensity of symptoms and the progression of the pathology (Ponnayyan Sulochana et al., 2014).

CELLULAR STRESS RESPONSE AND THE VITAGENE NETWORK

It is known that low concentrations of ROS are able to induce the expression of anti-oxidant enzymes and other defense mechanisms. The basis to explain this phenomenon refers to the concept of hormesis (Calabrese et al., 2006b), which is a dose–response relationship in which a given substance is stimulatory at low dose while at high doses exerts inhibitory effects. Radical active species may be beneficial since they act as signals to

enhance cellular defenses but become deleterious when present within a cells at high levels. ROS when in excess can over long-term disrupt redox homeostasis, cause oxidative stress, loss of molecular fidelity which underlie accumulation of unfolded or misfolded proteins in brain. Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, and Friedreich ataxia all belong to the so called "protein conformational diseases" affecting several thousands of aged people in all the world (Lodi et al., 2006). Unfolded protein response put in motion by chaperons actively rescue misfolded proteins, breaking up aggregates, and assisting the refolding process. Those proteins that cannot be rescued by refolding are, however, delivered to the proteasome by other chaperones and recycled (Lodi et al., 2006). In general, an unfolded protein response occur within conformational diseases characterized by dysfunctional aggregation of proteins accumulating in a non-native conformation. Under these physiopathological conditions, multiple metabolic derangements generally take place in association to excessive production of ROS and oxidative stress (Lodi et al., 2006).

The ability of a cell to withstand stressful conditions is defined cellular stress response (**Figure 1**). This phenomenon includes the HSR and represents a highly conserved ancient mechanism of cytoprotection (Calabrese et al., 2006b; Lodi et al., 2006). Synthesis of Hsps, which includes protein chaperones, is fundamental for proper folding and repair of denatured proteins, thus promoting conditions favorable to cell survival that would otherwise result in apoptotic cell death (Calabrese et al., 2006c; Scapagnini et al., 2006). Chaperones promote cell survival by sequestering damaged, denatured proteins and inhibiting formation of aggregates. The response to formation of aggregates is coordinated by an elaborated regulatory system which, simultaneously to up-regulation of several chaperones and other survival-promoting proteins, silences most cellular highly energy demanding functions. For this reason, most chaperones are also defined Hsps in reference to heat shock as the prototypic form of cellular stress. Chaperones under normal conditions elicit multiple roles, promoting proteins or RNA transport and remodeling events in large protein complexes essential for the regulation of cell migration, differentiation, signaling, transcription, and proliferation. Cellular stress response consist of pro-survival pathways controlled by cytoprotective genes called vitagenes (Mancuso et al., 2007b), resulting in the production of molecules endowed with anti-oxidant and anti-apoptotic potential, such as Hsps, glutathione, bilirubin (BR), and carbon monoxide (CO; Calabrese et al., 2007a). Vitagenes include members of the Hsp family, such as heme oxygenase-1 and Hsp72, sirtuins and the thioredoxin/thioredoxin reductase system (Calabrese et al., 2008d). Increasing evidence suggests that the HSR promotes cytoprotective conditions in several human disease states, such as mild chronic inflammation, cancer, aging, and neurodegenerative diseases (Calabrese et al., 2007b, 2008e, 2011). Thus, an emerging interest is growing on the cytoprotective potential of the HSR as pharmacological agents capable of inducing the HSR are important candidate for novel anti-degenerative and anti-inflammatory therapeutic strategies

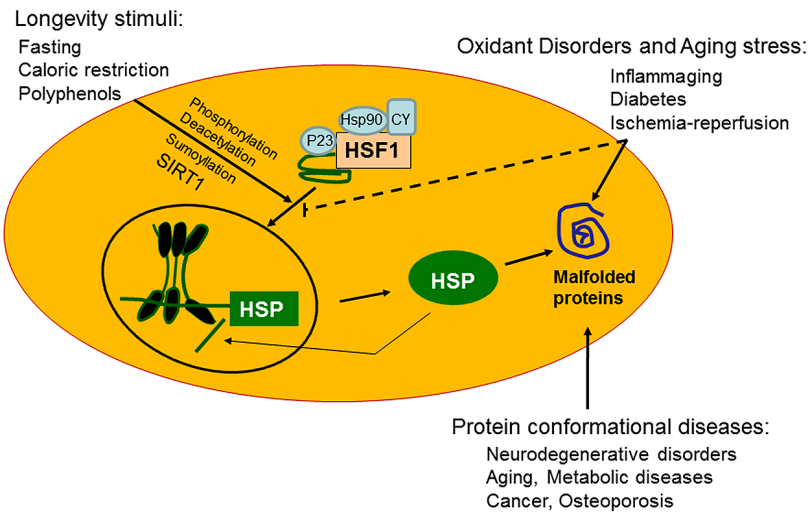

FIGURE 1 | Vitagenes and the pathway of cellular stress response. Misfolded proteins cumulating in response to proteotoxic stresses trigger the cellular stress response. Hsps that are normally bound to HSF1, maintaining it in a repressed state before stress, are titrate away by damaged or misfolded proteins with resulting HSF-1 activation. Multi-step activation of HSF1 involves post-translational modifications, such as hyperphosphorylation, deacetylation, or sumoylation, which allow HSF1 to trimerize, translocate into the nucleus, and bind to heat shock elements (HSEs) in the promoter regions of its target Hsp genes. Nutritional anti-oxidants, are able to activate vitagenes, such as heme oxygenase, Hsp70, thioredoxin reductase and sirtuins which represent an integrated system for cellular stress tolerance. Activation of vitagene system, with up-regulation of HO-1, thioredoxin, GSH, and sirtuin, results in reduction of pro-oxidant conditions. During inflammaging, including aged-associated pathologies, such as Alzheimer's disease and osteoporosis, a gradual decline in potency of the heat shock response occur and this may prevent repair of protein damage, leading to degeneration and cell death of critical parenchymal cells.

(Abdul et al., 2006; Mancuso et al., 2007a; Calabrese et al., 2008b,g,j).

Transcriptional, translational, and post-translational regulation of cellular stress response occur with the intervention of heat shock transcription factors (HSFs) normally expressed and maintained in an inactive state (Calabrese and Maines, 2006; Perluigi et al., 2006; Calabrese et al., 2008f,h; Zhang et al., 2014). Post-translational regulation of HSFs is an emerging area of interest integrating the metabolic state of the cell with biology of stress, and hence controlling important complex aspects of proteome molecular fidelity and physiology with consequent impact on aging processes. The HSR is transcriptionally controlled through *cis*-acting sequences called heat shock elements (HSEs) present in multiple copies upstream of the Hsp genes. Unlike invertebrates showing a single HSF, vertebrates express multiple HSFs as for plants. Four mammalian members of HSF have been identified: HSF1, HSF2, HSF3, and HSF4, all recognizing and binding the HSE, formed by inverted repeats of nGAAn consensus sequences. Thus, all four HSFs have overlapping functions associated with distinct patterns of tissue-specific expression, and undergo multiple post-translational modifications and exhibiting various interacting protein partners. Up-regulation of Hsp synthesis in response to stress, besides heat shock, is also initiated by redox-dependent mechanisms, endogenous or exogenous, in both cases resulting in trimerization and DNA binding of HSF1. Relevant to transactivation process is the cysteine moiety which operates as critical redox switch leading to the HSF activation (Zhang et al., 2014). In particular, formation of an intermolecular disulfide bond between cysteine 36 and cysteine 103 within HSF1, trigger trimerization and subsequent DNA binding, whereas a disulfide intramolecular bond formation has inhibitory effects for the transactivating activity of HSF. Consistent with this notion, modulation of HSF-mediated gene regulation has an emerging pharmacologic potential, as small-molecule with the capability of activation or inhibition of HSFs, can exert important influence on aging, neurodegenerative processes and longevity mechanisms (Fujimoto et al., 2010; Zhang et al., 2011; Akerfelt et al., 2012; Westerheide et al., 2012). The regulatory domain of HSF has competence to sense heat shock, however, its induction is post-translationally controlled (Fujimoto et al., 2010; Akerfelt et al., 2012; Westerheide et al., 2012). In normal non-stressed cellular conditions, HSF1 is maintained inactive and in a monomeric state through interaction with Hsp90. Thus, pharmacological inhibition of Hsp90 results in a conversion of monomeric HSF1 in a trimerized HSF1 with DNA-binding competence (Calabrese et al., 2008c,i, 2009a,b,d). Trimerization, nuclear translocation and interaction with HSE in the DNA rapidly declines in cells soon after proteotoxic insult exposure, since HSF1 binding to the promoter occurs within seconds, reaching in about 1 min saturation with the contribution of other components such as a ribonucleoprotein complex containing the eukaryotic elongation factor 1A and a constitutive non-coding RNA, heat shock RNA-1 (HSR-1) endowed with heat-sensing capability (Calabrese et al., 2009c, 2010g,h). According to the most accredited model, in response to heat shock HSR-1 undergoes conformational changes facilitating HSF1 trimerization. Under this transactivating conditions, HSF1 is post-translationally modified through phosphorylation, sumoylation, and/or acetylation (Calabrese et al., 2010d, 2011). Increasing evidence suggests that phosphorylation and sumoylation of HSF1 is a phenomenon occurring rapidly after heat shock, while acetylation occurs with a delayed kinetics in coincidence of the attenuation phase of the cycle of HSF1 activation. In particular, acetylation of HSF1 is a process regulated by the ratio of acetylation/deacetylation controlled by p300–CBP (CREB-binding protein) and by the NAD^+-dependent sirtuin, SIRT1. With respect to this, enhanced DNA-binding activity of HSF1 ensues after increased expression and activity of SIRT1, whereas acetylation of HSF1 consequent to down-regulation of SIRT1 reduces DNA-binding activity with no effect on the trimerization process (Calabrese et al., 2010d). Increasing evidence indicates the involvement of SIRT1 in caloric restriction and aging, as demonstrated in senescent cells where the age-dependent loss of SIRT1, associated to impairment in HSF1 activity, correlate with attenuation of the HSR and disruption of proteostatic mechanisms, thereby linking HSR to nutrition and aging (Calabrese et al., 2010e,f; Di Paola et al., 2011).

Keap1/Nrf2/ARE BIOLOGY AND THE HEME OXYGENASE PATHWAY OF STRESS TOLERANCE

HSF1 is a central regulator in the gene expression of Hsps, however, in addition to this mechanism controlling vitagenes coordination, a response protecting against various electrophiles and oxidants operates integrating cytoprotection as "phase 2 response," known also as "the electrophile counterattack response" (Zhang et al., 2011, 2014). Phase 2 response proteins include heme oxygenase 1, thioredoxin, and thioredoxin reductase, all of which can be up-regulated by the transcription factor Nrf2 (nuclear erythroid 2-related factor 2), similarly to other cytoprotective proteins, including NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamylcysteine synthetase, glutathione reductase, glutathione transferases (GST), UDP-glucuronosyltransferase, epoxide hydrolase, aldo-keto reductases, ferritin, and glutathione conjugate efflux pumps (Zhang et al., 2011, 2014). The elaborate network of Nrf2-dependent phase 2 response proteins enable eukaryotic cells to counteract the damaging effects associated to oxidants and electrophiles, agents primarily involved in the pathogenesis of neurodegenerative disorders, atherosclerosis, aging, and cancer (Zhang et al., 2011, 2014). A major factor operating in the control of the expression of these proteins is the Keap1/Nrf2/ARE system. Activation of gene expression requires that Nrf2, a basic leucine zipper transcription factor, binds in the upstream regulatory regions of these genes containing single or multiple copies of the anti-oxidant/electrophile response elements (ARE, EpRE). Nrf2 binding to the ARE occur in heterodimeric combinations with members of the small Maf family of transcription factors. Under normal conditions the pathway operates at basal levels controlled by a cytosolic protein with function of repressor, the Kelch-like ECH-associated protein 1 (Keap1). When Keap1 is bound to Nrf2, the latter is presented, by binding to the E3 ubiquitin ligase Cullin3–RING box1 (Cul3–Rbx1), to the ubiquitin system for proteasomal degradation. At least 10 distinct chemical classes of inducers of the Keap1/Nrf2/ARE pathway, are known and belong to: (i) Michael acceptors (olefins or acetylenes

conjugated to electron-withdrawing groups); (ii) isothiocyanates; (iii) oxidizable diphenols, quinones, and phenylenediamines; (iv) thiocarbamates; (v) trivalent arsenicals; (vi) dithiolethiones; (vii) hydroperoxides; (viii) vicinal dimercaptans; (ix) heavy metals; and (x) polyenes. All these members share the common property of presenting chemical reactivity toward sulfhydryl groups through oxido-reduction, alkylation, or disulfide interchange mechanisms (Calabrese et al., 2010d, 2011; Zhang et al., 2011, 2014).

Heme oxygenase is a sensors of oxidative stress within cells modulating redox status and homeostasis. Heme oxygenase-1, an Hsp32, protects against oxidative damage converting pro-oxidant heme into biliverdin, free iron, and CO. With the intervention of the enzyme biliverdin reductase (BVR) biliverdin is then reduced by into BR, a linear anti-oxidant tetrapyrrole (Calabrese et al., 2010d). BR has been demonstrated efficiently buffer nitrosative stress, owing to its capability to bind and inactivate NO-derived RNS (Pennisi et al., 2011; Scapagnini et al., 2011). Two HO isoforms are known: an inducible isoform, HO-1 and HO-2 which is a constitutive enzyme (Calabrese and Maines, 2006). These isoforms share only 43% homology being products of different genes. HO-1 and HO-2 present important differences in cell and tissue regulation and distribution (Mancuso et al., 2006, 2008). Moreover, only in rat as third protein, HO-3 has been found to be retrotransposomal product of the HO-2 gene (pseudogene). In spite of playing the same enzymatic role, HO-1 and HO-2 have different protective function in tissues. HO-1 induction occurs as early response to oxidative insult resulting rapid transformation of heme into CO and BR. On the contrary, constitutive HO-2, regulates heme homeostasis a function associated within a cell with the sensing of intracellular levels of CO. HO-1, is induced in response to oxidative and nitrosative stress, heme, Aβ, dopamine analogs, H_2O_2, hyperoxia, UV light, heavy metals, prostaglandins, NO, peroxynitrite, Th1 cytokines, oxidized lipid products, and lipopolysaccharide, as well as growth factors. In all these conditions, the ARE contained in its promoter region, is central to its redox regulation (Alam and Cook, 2007; Calabrese et al., 2012b), recognizing two upstream enhancers, E1 and E2 where multiple ARE elements are contained. Consistently, polymorphisms in the lengths of GT repeats (Calabrese et al., 2011; Pennisi et al., 2011; Scapagnini et al., 2011) within the *ho-1* promoter is critical for the expression and functions of HO-1 in humans. Higher HO-1 activity is associated with the short-GT polymorphisms, a conditions thought be protective against oxidant disorders (e.g., coronary artery disease, atherosclerosis-linked conditions), whereas long GT sequences, coding for a relatively unstable, Z-conformational DNA, exhibits reduced transcriptional activity, as well as basal and stimulated HO-1 protein levels, as found in various malignant conditions (Calabrese et al., 2010d). Overexpression of HO-1 results in increased cGMP and bcl-2 levels in neurons, inactivation of p53, up-regulation of anti-oxidant proteins as well as ferritin which sequesters and inactivates free iron (Alam and Cook, 2007; Calabrese et al., 2012b). Although large consensus exists on the fact that increased HO-1 expression is a protective mechanism operating under oxidative stress conditions, however, there is evidence that HO-1 can be repressed during the same conditions, particularly during hypoxia. HO-1 repression under this condition is sustained by the heme-regulated transcription factors Bach1/Bach2 (Calabrese et al., 2010d, 2011) and it has been argued that this might be necessary to reduce the energy request due to heme degradation, to limit formation of CO and BR which can accumulate to toxic levels, and to increase heme availability for proper mitochondrial respiration (Calabrese et al., 2006c, 2008g). Neurodegeneration as a chronic inflammatory oxidative condition caused by Aβ metabolic disruption, makes HO-1 an important target for therapeutic anti-inflammatory strategies. HO-1 mRNA and protein expression have been documented to be increased in AD brain, in association with neurofibrillary tangles. This finding may have the biological meaning of a protective response in vulnerable neurons to transform pro-oxidant heme into BR and CO, whose anti-oxidant and anti-inflammatory nature has been ascertained. This opens new therapeutic avenue, as polyphenols contained in herbs and spices can represent potential drugs for the prevention and treatment of AD (Scapagnini et al., 2006; Calabrese et al., 2007a, 2008b). Epidemiological studies have demonstrated that curcumin is responsible for the significantly reduced (4.4-fold) prevalence of AD in India, compared to United States (Calabrese et al., 2007b, 2008b; Mancuso et al., 2007a), and curcumin given chronically in the diet to transgenic APPSw, a mouse model (Tg2576) of AD suppresses brain inflammatory and oxidative damage, an effect associated to inhibition of nuclear factor-κB (NF-κB) and efficient prevention of neuronal cell death (Calabrese et al., 2009b,d, 2010d,h; Gupta et al., 2011).

HORMESIS

Hormesis is a dose–response phenomenon, characterized by a low-dose stimulation and a high-dose inhibition. The term hormesis was first introduced into the scientific literature in 1943 by Chester Southam and John Ehrlich, mycology researchers at the University of Idaho, who reported that low concentrations of extracts from the Red Cedar tree enhanced the metabolism of a number of fungal species. The term hormesis was derived from the Greek meaning to excite. Prior to the report of Southam and Ehrlich (1943), there was a substance history of reports in the biological literature on also demonstrating a similar biphasic dose response. It was first reported by Schulz who found that low concentrations of numerous disinfectants stimulated metabolism at low concentrations while being inhibitory at higher concentrations (Schulz, 1887, 1888). The research of Schulz was confirmed by other investigators and extended to other biological models and agents, with particular emphasis on bacteria, fungi, and plants over the next several decades. The phenomenon was initially referred to as the Arndt-Schulz Law and Hueppe's Rule, with these terms being subsequently replaced by the term hormesis. The occurrence of hormetic dose responses from the time of Schulz up to approximately 1950 has been summarized in depth for both chemical and radiation induced hormesis (Calabrese and Baldwin, 2000a,b,c,d,e).

Despite the fact that the hormesis concept has considerable historical literature supporting its occurrence and reproducibility, it failed to gain traction within the biomedical community due

to the fact that Schulz inappropriately claimed that he had discovered the explanatory principle of homeopathy, based on his biphasic dose response with yeast (Calabrese, 2005). This proclamation led to strong disputes with medically oriented researchers principally due to the long and intense conflict between homeopathy and traditional medicine. In fact, a considerable effort by leading pharmacologists of the Schulz era, such as Alfred J. Clark, devoted considerable effort to criticize Schulz and his biphasic dose response and to try to link it with high dilution homeopathy, in an effort to marginalize and discredit homeopathy (Calabrese, 2011). In addition to such historical antipathies, the concept of hormesis was also difficult to prove scientifically as it required a substantial number of doses, especially in the low dose zone, reasonably high statistical power, and biological model systems with generally low variability and the strong capacity for replication of findings. These factors are important since the low dose stimulation is modest, with most maximum stimulatory responses only being about 30–60% greater than the unexposed control group (**Figure 2**). Thus, trying to test hormetic hypotheses requires far more rigorous study designs and more resources than was usually the case in hazard assessment type investigations.

As a result of the historical conflicts between homeopathy and traditional medicine and the inherent challenges in testing hormetic hypotheses, the concept of hormesis never became well established as a scientific concept in throughout most of the twentieth century (Calabrese, 2008d). However, since the late 1970s there has been a multi-disciplinary-based resurgence of the hormesis concept, based largely on technical improvements in the capacity to measure at lower concentrations, advances in *in vitro* and high throughput research which has facilitated the capacity to test a wide range of concentrations in an efficient manner, the development of mechanistic understandings of biphasic dose responses especially at the receptor and cell signaling pathways levels and the dose response and mechanistic linkage of developments in the area of adaptive responses, including that of preconditioning with hormesis (Calabrese, 2010).

Within recent years substantial hormetic dose response data bases have been developed based on rigorous *a priori* entry and evaluative criteria providing documentation for the hormetic dose responses (Calabrese and Blain, 2005, 2009, 2011). These databases provide strong evidence that hormetic dose responses are independent of biological model, endpoint measured, the chemical class of the inducing agent, the level of biological organization (i.e., cell, organ, organism) and mechanism (Calabrese, 2013d). These databases have confirmed and far more firmly established the quantitative features of the hormetic dose response, revealing that it represents a modest stimulation, usually less than twice the value of the control group. It has also been established that the hormetic dose response is typically reported for highly integrated endpoints, especially those relating to cell proliferation, complex behaviors, adaptive/preconditioning responses, reproductive endpoints such as fecundity, cancer, mutation, cancer endpoints, and longevity/aging. Detailed evaluations of the pharmaceutical literature have also revealed that entire drug classes are based up the hormetic dose responses. For example, preclinical studies in the areas of anxiolytic drugs (Calabrese, 2008b), anti-seizure drugs (Calabrese, 2008e), memory enhancing agents (Calabrese, 2008a), drugs treating osteoporosis (Calabrese, 2008c), and others typically demonstrate hormetic dose responses. Since

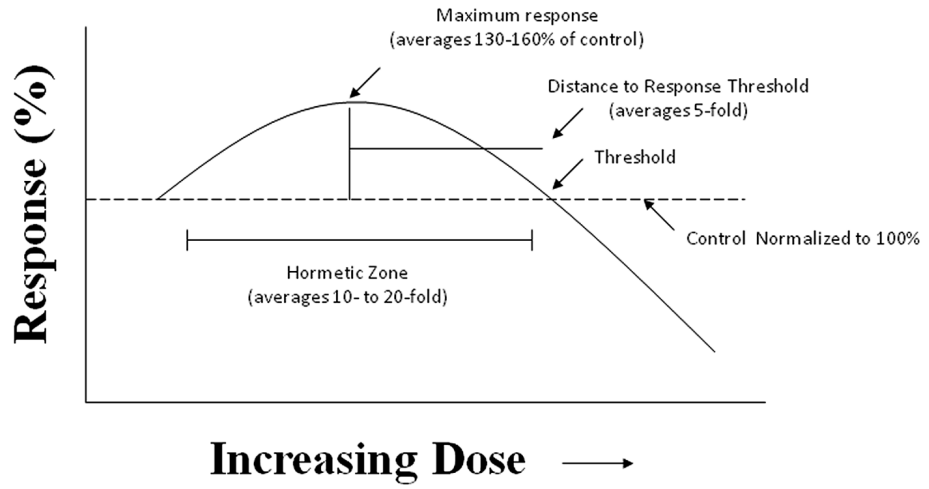

FIGURE 2 | The hormesis (biphasic) dose–response curve: generalized quantitative features. The hormetic dose response is characterized by specific quantitative features concerning its amplitude and width. Based on more than 10,000 hormetic dose responses in various hormetic databases, approximately 80% have their maximum response less than twofold greater than the control. The strong majority of hormetic dose responses extend over approximately a 5- to 10-fold dose range immediately below the estimated response threshold. The hormetic dose response may occur either as a result of a direct stimulation or as an overcompensation response following an initial disruption in homeostasis, in both cases being the quantitative features similar. The hormetic response appears to quantitatively describe the limits of biological plasticity across phyla as well as at different levels of biological organization (cell, organ, and organism). The quantitative features of the hormetic dose response are also independent of mechanism. Pharmaceutical treatments for a wide range of human conditions are based upon the hormetic biphasic dose response as discovered within preclinical evaluations and then directly applied to human populations.

it is now believed that the modest hormetic stimulation is a measure of biological plasticity it suggests that the hormetic dose responses describes the degree to which pharmaceutical agents can enhance biological performance (Calabrese and Mattson, 2011; Calabrese, 2013a). It is important to note that during the entire twentieth century the biomedical and regulatory communities never validated the capacity of the threshold dose response to make accurate predictions below the threshold, that is, the area of the dose response where most people spend the majority of their time. This lead to a series of direct, head-to-head comparisons between the threshold, linear and hormetic dose response models using multiple large and independent dose response data sets with multiple models, endpoints and agents (Calabrese and Baldwin, 2001, 2003; Calabrese et al., 2006a, 2008a, 2010a). In each of these assessments, the hormetic dose response significantly out-performed the threshold and linear dose response models.

Major comprehensive evaluations of the biomedical literature have revealed that hormesis dose responses are commonly reported in essentially all areas of research including the immune system, tumor cell biology, neuroscience, including memory, stress, anxiety, seizure, pain, numerous degenerative diseases, wound healing and for a very broad range of receptor systems and peptides (Calabrese, 2013b). The applications of the hormesis dose response are therefore extensive, affecting drug discovery, drug development, and the design of the clinical trial. Furthermore, even areas such as preconditioning are based on the hormetic concept (Calabrese et al., 2010b,c, 2012a, 2013; Calabrese and Calabrese, 2013a,b; Krenz et al., 2013). This may be seen in numerous studies which have assessed a wide range of graded preconditioning doses with results being reflective of the biphasic-hormetic dose response. The historical assessment of the dose response has revealed that the dose response model that was once rejected by the biomedical community about a century ago is now the dose response model that has repeatedly outcompeted the traditional doses responses models in direct comparisons. It is the dose response model that has provided quantitative insight into the magnitude of biological plasticity and the theoretical basis for it. The hormetic dose response thus underlines the basis of pharmaceutical strategies aimed at enhancing biological performances in a broad range of scientific areas.

BONE REMODELING, REDOX STATE, AND Aβ METABOLISM

Osteoporosis is a devastating disease having enormous health and economic impacts, particularly considering the global shift toward an aging population, characterized by a systemic and progressive skeletal pathology characterized by compromised bone mineral density and strength with the increased occurrence of fractures. Despite rapid progress in our understanding over recent years, patient morbidity and mortality resulting from this disease are still too high (Ono et al., 2014), and there is an urgent need for a proper assessment of the underlying mechanisms and the development of new treatment strategies to address this pathophysiological issue. Patients with AD show significantly increased risk of osteoporotic hip fractures. However, whether abnormal Aβ peptide (Aβ) deposition also occurs in osteoporosis and the relationship between Aβ and human osteoporosis remains an open, not elucidated, question (Li et al., 2014).

Amyloid-β peptide, one of the pathological hallmarks of AD, is a small (40–42 amino acids) proteolytic fragment of a glycosylated integral membrane cell surface receptor protein called APP and is encoded by a gene on human chromosome 21 (Liu and Chan, 2014; Richard and Brayne, 2014). Aβ has attracted much attention for its association with various pathologies (Stefanova et al., 2014). Besides AD, Aβ plays a crucial role in other important neurodegenerative diseases, such as Huntington's, Parkinson's, and prion disorders, as well as amyotrophic lateral sclerosis as well as type 2 diabetes and the most common age-related muscle disease of inclusion body myositis (Tóth et al., 2014). Thus, APP/Aβ seems to be associated with multiple degenerative disorders. Epidemiological studies showed that patients with AD had an increased risk of developing osteoporotic hip fractures even after considering the increased frequency of fallings in AD patients (Xia et al., 2013), suggesting one or more common denominators between both disorders. Nevertheless, an association between Aβ and human osteoporosis has not yet been clearly established, and also it has been inferred that Aβ may be of physiological importance for survival of cells (Li et al., 2014). Excessive Aβ aggregates and fibrillates to form amyloid plaques in the brain, thus leading to the exacerbation of AD pathology. Previous studies have identified a role for Aβ in the activation of osteoclasts through gene knockout experiments and use of the transgenic AD mouse model, Tg2576 (Li et al., 2014). However, whether a large amount of Aβ deposits also occur in osteoporotic bone tissues and the role human Aβ may play on OC activation remain unclear. In addition to having an activation effect on osteoclasts, Aβ may accumulate abnormally in osteoporotic bone and play an important pathogenic role. A close relationship between Aβ and osteoporosis is shown across species from rodent to human, as demonstrated in different clinical conditions in patient samples as well as in various animal model and cell cultures. AD and osteoporotic hip fractures often coexist during aging. Platelets have been shown to be the primary source (90%) of Aβ in human blood with plasma Aβ levels fluctuating over time among individuals (Roher et al., 2009). Besides human plasma and cerebrospinal fluid, soluble Aβ is also a component of human urine in Alzheimer's. Despite this level of knowledge surrounding APP and Aβ expression in many tissues, reports on the expression and distribution of Aβ in bone tissues and osteocytes remain an emerging evidence. Aβ deposition has been found on the endosteal and periosteal surfaces of adult rat ulnae (Li et al., 2014). Notably, occurrence of Aβ and APP abnormal accumulation in different tissues supports the hypothesis that Aβ diseases may be a systemic disease, suggesting that these malfolded proteins may either be produced locally in diverse organs or may originate from a common circulating precursor. Consistent with this notion, abnormal Aβ and APP burden has been detected in osteoporotic bone tissues from both human and rat OVX models, where Aβ42 was identified mainly in the membrane and cytoplasm of osteocytes and extracellular matrix, while APP largely found in the membrane of osteocytes. Despite increasing research efforts, still the mechanism underlying the accumulation of Aβ and APP in osteocytes in osteoporotic bones remains elusive. One possible source for

Aβ deposition in bone may be blood, where Aβ increases during senescence (Li et al., 2014). In addition to this, secretion of Aβ by mature osteoblasts has been documented, in agreement with the finding of Aβ42 and APP formation in osteoblasts from both human and OVX rats osteoporotic bone. In these conditions, APP has been found to be able of suppressing osteoblast differentiation, associated with osteoporotic alterations (Xia et al., 2013). Given that osteoblast is the precursor of the osteocyte, it is conceivable that the deposition of Aβ and APP in osteocytes can be consequence of secretion by osteoblasts during both osteogenic differentiation and aging processes. In turn, accumulating Aβ may promote apoptotic process in osteocytes, likewise in neurons thus determining bone loss and osteoporosis (Cui et al., 2011). An interesting and controversial question concerns why the abundant presence of proteins of Aβ abnormal metabolism in the bone of osteoporotic patients did not cause them to develop brain degeneration, such as AD. A number of explanations may exist to provide a possible rationale. First, bone is a very special organ, with limited blood supply, with most of the osteocytes embedded in the matrix without direct contact with blood. In these conditions, release of Aβ into the blood stream is not an easy process. Second, the blood–brain-barrier (BBB) permeability can be of extreme importance for the prevention of Aβ invasion into the brain tissues, as uptake of peripheral Aβ by the brain is not a normal occurrence without BBB compromission (Jefferies et al., 2013). Lastly, both osteoporosis and AD are multifactorial diseases with complex etiology and pathogenesis (Rachner et al., 2011). Aβ deposits are present in several tissues, which indicates that the protein may originate as product of local metabolism in various organs or, similarly to other amyloidoses, can derive from a circulating precursor common to all these pathophysiological conditions. However, further studies are necessary to explore these dynamics and understand the underlying mechanisms. Abnormal Aβ deposition in osteoporotic bone tissues and its potent enhancement effect on osteoclast differentiation and activation, is already clearly demonstrated suggesting an important role for Aβ in the pathogenesis of osteoporosis (Li et al., 2014). This is of great clinical significance for providing novel insights into the tight link between Aβ and human osteoporosis, thus revealing a potential mechanism underlying altered bone mineral density by Aβ abnormal metabolism. Clearly, however, further work is required to elucidate the exact mechanisms through which Aβ regulates osteoporosis signaling. These research efforts may eventually lead to a promising future discovery of a new etiology for osteoporosis, and prompt healthcare professionals and researchers to develop innovative anti-bone-resorptive therapeutic agents and strategies, particularly those designed by targeting Aβ, to efficiently minimize deleterious consequences associated with bone homeostasis disruption. In line with these evidence, since a biomarker is a traceable substance indicating changes in expression or metabolism of a given protein which correlates with the risk or progression of a disease, as consequence, Aβ may be a novel and promising candidate biomarker for drug targeting and characterization of osteoporotic therapeutic approaches in the future (Osorio et al., 2014).

Bone tissue undergoes, throughout life, a continuous renewal through a process called bone remodeling, which is controlled by the activity of osteoclasts mediating bone resorption and parallel activity of osteoblasts which mediate bone formation (Vacek et al., 2013). Any disturbance in the balanced formation and resorption process, which can be linked to hormone disequilibrium or aging decreases bone mass and result in bone pathologies, such as osteoporosis leading to increased vulnerability to fractures. Within this context, the receptor activator of NF-κB ligand (RANKL) appears to be an important factor underlying osteoporosis pathogenesis for its critical role played in osteoclast differentiation and activation (Park et al., 2014). For this reason, inhibition of RANKL represents an innovative therapeutic target for controlling osteoclastogenesis (Park et al., 2011). Notably, an important role in bone remodeling is played by alternative or non-canonical NF-κB pathway, which mediates activation of the p52/RelB NF-κB complex, thus regulating various biological processes. This pathway differently from IκBα degradation in the canonical mechanism, consists of processing of p100 a NF-κB2 precursor protein,. In this context a central role is played by NF-κB-inducing kinase (NIK), a component of the non-canonical NF-κB pathway and a downstream kinase, IKKα (inhibitor of NF-κB kinase) which operate with integrated functions promoting induction of phosphorylation-dependent ubiquitination of p100. Under normal conditions, NIK is processed by a tumor necrosis factor (TNF) receptor-associated factor-3 (TRAF3)-dependent E3 ubiquitin ligase. After signals mediated by a subset of TNF receptor superfamily members, TRAF3 is degradated and NIK is stabilized leading to non-canonical activation of NF-κB (Sun, 2012; **Figure 3**). Accordingly, the inhibitory role of p100, in both basal and stimulated osteoclastogenesis in bone formation as well as resorption has been clearly demonstrated (Soysa et al., 2010). In the alternative NF-κB pathway p52 derived from p100 through NIK, binding of p52 and RelB induces effects on osteoclast biology (Soysa et al., 2010). However, to date, the precise physiologic importance of alternative NF-κB in bone biology, is not completely elucidated. Furthermore, the currently known intracellular signaling pathways activated after receptor binding of RANKL include the nuclear factor of activated T cells (Piva et al., 2009), mitogen-activated protein kinases (MAPKs), TRAFs, c-Jun N-terminal kinases (JNKs), and ROS (Kaunitz and Yamaguchi, 2008; Kanzaki et al., 2013). In addition, NF-κB is a transcription factor, which pleiotropically regulate osteoclast formation, function, and survival (Piva et al., 2009). Deletion of both NF-κB p50 and p52 subunits is associated to osteopetrosis as consequence of osteoclast absence and, in addition, NF-κB is central for the differentiation of RANK-expressing osteoclasts into osteoclasts $TRAP^{+}$ induced by osteoclastogenic cytokines. This explain the inhibitory effect on osteoclast formation induced by prevention of NF-κB activation (Piva et al., 2009; Augustine and Horwitz, 2013).

Reactive oxygen species act as intracellular signaling molecules involved in the regulation of RANKL-dependent osteoclast differentiation, but they also have cytotoxic effects that include peroxidation of lipids and oxidative damage to proteins and DNA. Taking into account the relationship between Nrf2 and osteoclastogenesis, stimulation of osteoclast precursors (mouse primary peritoneal macrophages and RAW 264.7 cells) with RANKL results in the up-regulation of Keap1, a negative regulator of Nrf2, with decreased

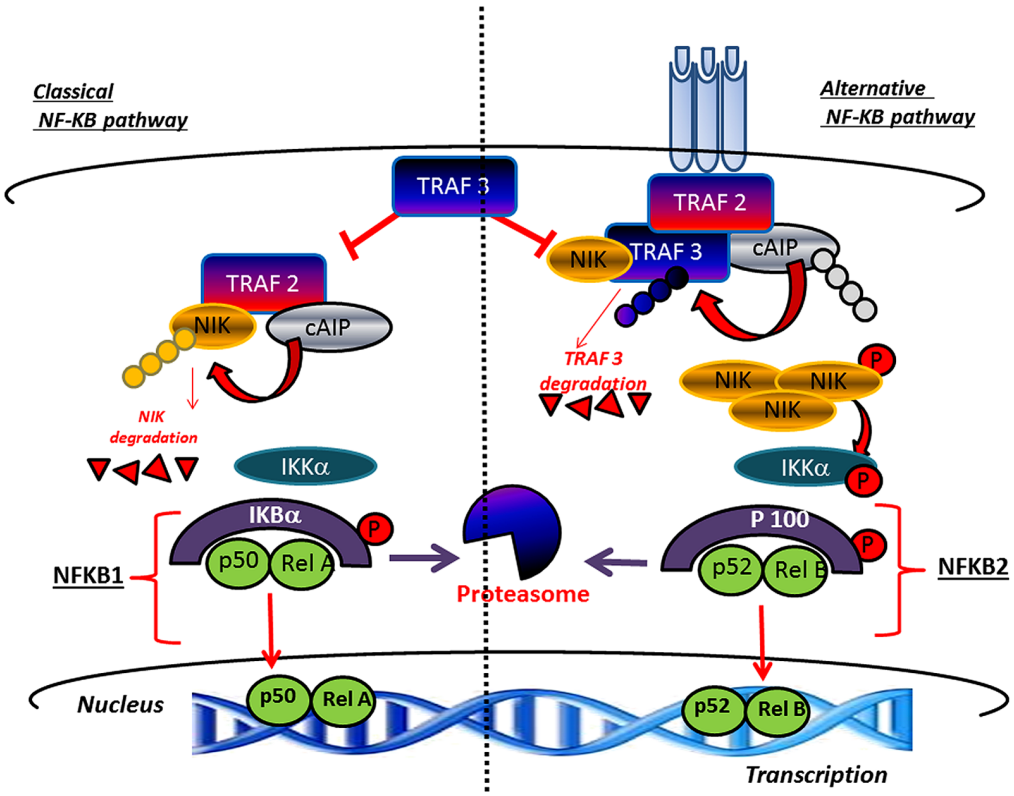

FIGURE 3 | Canonical and non-canonical pathway leading to the activation of NF-κB. TRAF3 inhibits activation of the classical NFκB pathway A high level of TRAF3 interferes with the recruitment of TRAF2 to the receptor. In parallel, TRAF3 induces NIK degradation and consequently inhibits the activation of the alternative arm of NFκB. The activation of canonical pathway results in the phosphorylation of IκBαα by the IKK complex, leading to its ubiquitylation and subsequent degradation by the proteasome. The RelA/p50 complex is free to translocate to the nucleus to activate the transcription of target genes. The non-canonical pathway results in the NIK stabilization. In response to receptor crosslinking, TRAFs and cIAP1/2 are recruited to the receptor, where cIAP1/2 ubiquitinates TRAF2 and TRAF3 and stimulates their degradation. Accumulated NIK activates IKKα, which in turn phosphorylates p100, leading to p100 processing to p52, which can lead to the activation of p52-RelB that target distinct κB element and induce the transcription of target genes.

Nrf2/Keap1 ratio, and down-regulation of cytoprotective enzymes, such as heme oxygenase-1 and γ-glutamylcysteine synthetase (Kanzaki et al., 2013). On the other hand, Nrf2 overexpression results in up-regulation of the expression of cytoprotective enzymes, associated with decrease in ROS levels, tartrate-resistant acid phosphatase-positive multinucleated cell number, as well as osteoclast differentiation, and attenuation of bone destruction, as found both *in vitro* and *in vivo* models (Kanzaki et al., 2013). Consistent with this line of evidence, overexpression of Keap1 or RNAi-induced knock-down of Nrf2 resulted in effects opposite to those obtained by stimulation of Nrf2-dependent DNA binding activity (Kanzaki et al., 2013). The precise mechanisms by which stimulation with RANKL reduces Nrf2 is not currently known. It is known Keap1 has highly reactive thiol groups in its structure and that oxidation of this domain leads to significant changes in the conformation of Keap1, resulting in dissociation from Nrf2 and stimulation of nuclear Nrf2-dependent DNA binding activity (Kanzaki et al., 2013). In addition, Nrf2 (see previous section) autoregulates its own expression (Calabrese et al., 2010d; Zhang et al., 2011, 2014). Taken together, this evidence implies that an increase in ROS levels induced by stimulation with RANKL may up-regulate Nrf2. It has also been reported that Nrf2 regulates Keap1 by controlling its transcription (Calabrese et al., 2010d; Zhang et al., 2014). Change of stability of Nrf2 mRNA or decrease of translation by miRNA can modulate RANKL-dependent Nrf2 down-regulation. Also, Bach1, an inhibitor of Nrf2 binding to the ARE, could participate to this mechanism, as indicated by attenuated osteoclastogenesis found in Bach1 knock-out mice (Kanzaki et al., 2013). However, although extensive investigations will be required to clarify the exact regulatory mechanisms linking Nrf2 to stimulation with RANKL, it is clearly proven that Keap1/Nrf2 axis regulates RANKL-dependent osteoclastogenesis through redox-modulation of intracellular ROS signaling and expression of cytoprotective enzymes. This raises the exciting possibility that the Keap1–Nrf2 axis may be a therapeutic target for the treatment of bone destructive disease.

CONCLUSION

Comprehensive evaluation of the biomedical literature has pinpointed the role of hormetic dose responses as reported in essentially all areas of research, including the immune system,

tumor cell biology, neuroscience, drugs treating osteoporosis, as well as wound healing and a broad range of receptor systems and peptides (Calabrese, 2008c, 2013b). Applications of the hormesis dose response are therefore extensive, affecting drug discovery, drug development, and the design of the clinical trial. It is now conceivably expected that the hormetic concept is at the core of attempts leading to enhanced development of biological shields via processes such as preconditioning which can help to improve the quality normal living, the aging process and to reduce the impact of various types of degenerative diseases. AD is a chronically progressive disorder, in which neurodegenerative damage progresses for many years before clinical onset (Calabrese et al., 2006d; Liu and Chan, 2014; Richard and Brayne, 2014). Osteoporosis is a progressive degenerative disorder, also recognizing a multifactorial origin that is characterized by bone deterioration resulting in fragility and fractures. The medical and social and economic effects of osteoporosis, given the increasingly aging population, in particular osteoporosis of postmenopausal nature, will growth drastically. A better knowledge of bone biology with particular regard to the functional relationship between bone-formation by osteoblast activity and bone-resorption by osteoclasts with a better definition of the underlying signaling network has unraveled novel therapeutic targets. Osteoporosis and hip fracture are commonly observed complications seen in patients with AD. Although the mechanisms underlying this association remain poorly understood, emerging evidence supports the view that AD risk genes may also be a risk factor for osteoporosis, and that AD and osteoporosis may share conserved oxidative stress-driven pathogenic mechanisms. Consistent with this notion, using transgenic mice expressing an AD-associated mutant form of APP, known as APPswe, to explore the effects of mutant APP on bone remodeling, significant defects in bone formation associated with reduced differentiation of bone marrow stromal cells into osteoblasts in *ex vivo* culture experiments, were found in these mice compared with wild-type controls. Treatment with *N*-acetylcysteine ameliorated the impact of APPswe on bone marrow stromal cell differentiation, and increased bone volume in these transgenic mice (Woodman, 2013). Thus, ROS may be a conserved mechanism underlying APPswe-induced neurodegenerative and osteoporotic pathological alterations. Bone remodeling is a process of continuous formation and resorption occurring in specific areas of the matrix. Novel therapeutic strategies have been developed focused on the inhibition of excessive bone resorption and promotion of bone formation process. Due to novel drugs available, using the most recent knowledge of bone-cell biology, we have increased our therapeutic intervention to osteoporotic patients with more individualized lines of treatment. Accordingly, basic research can greatly contribute to the identification of specific pathways that can be effectively targeted by novel compounds able to treat and possibly reverse osteoporosis, particularly that occur in already chronically severed patients, such as in neurodegenerative disorders.

REFERENCES

Abdul, H. M., Calabrese, V., Calvani, M., and Butterfield, D. A. (2006). Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. *J. Neurosci. Res.* 84, 398–408. doi: 10.1002/jnr.20877

Abramov, E., Dolev, I., Fogel, H., Ciccotosto, G. D., Ruff, E., and Slutsky, I. (2009). Amyloid-beta as a positive endogenous regulator of release probability at hippocampal synapses. *Nat. Neurosci.* 12, 1567–1576. doi: 10.1038/nn.2433

Akerfelt, M., Vihervaara, A. A., Laiho, A., Conter, A., Christians, E. S., Sistonen, L., et al. (2012). Heat shock transcription factor 1 localizes to sex chromatin during meiotic repression. *J. Biol. Chem.* 285, 34469–34476. doi: 10.1074/jbc.M110.157552

Alam, J., and Cook, J. L. (2007). How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am. J. Respir. Cell Mol. Biol.* 36, 166–174. doi: 10.1165/rcmb.2006-0340TR

Augustine, M., and Horwitz, M. J. (2013). Parathyroid hormone and parathyroid hormone-related protein analogs as therapies for osteoporosis. *Curr. Osteoporos. Rep.* 11, 400–406. doi: 10.1007/s11914-013-0171-2

Begum, A. N., Jones, M. R., Lim, G. P., Morihara, T., Kim, P., Heath, D. D., et al. (2008). Curcumin structure–function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J. Pharmacol. Exp. Ther.* 326, 196–208. doi: 10.1124/jpet.108.137455

Bellia, F., Calabrese, V., Guarino, F., Cavallaro, M., Cornelius, C., De Pinto, V., et al. (2009). Carnosinase levels in aging brain: redox state induction and cellular stress response. *Antioxid. Redox Signal.* 11, 2759–2775. doi: 10.1089/ars.2009.2738

Bellia, F., Vecchio, G., Cuzzocrea, S., Calabrese, V., and Rizzarelli, E. (2011). Neuroprotective features of carnosine in oxidative driven diseases. *Mol. Aspects Med.* 32, 258–266 doi: 10.1016/j.mam.2011.10.009

Borger, E., Aitken, L., Du, H., Zhang, W., Gunn-Moore, F. J., and Yan, S. S. (2013). Is amyloid binding alcohol dehydrogenase a drug target for treating Alzheimer's disease? *Curr. Alzheimer Res.* 10, 21–29.

Bukanova, J. V., Sharonova, I. N., and Skrebitsky, V. G. (2014). Amyloid β peptide (25–35) in picomolar concentrations modulates the function of glycine receptors in rat hippocampal pyramidal neurons through interaction with extracellular site(s). *Brain Res.* 1558, 1–10. doi: 10.1016/j.brainres.2014.02.031

Calabrese, E. J. (2005). Historical blunders: how toxicology got the dose–response relationship half right. *Cell. Mol. Biol.* 51, 643–654.

Calabrese, E. J. (2008a). Alzheimer's disease drugs: an application of the hormetic dose–response model. *Crit. Rev. Toxicol.* 38, 419–451. doi: 10.1080/10408440802003991

Calabrese, E. J. (2008b). An assessment of anxiolytic drug screening tests: hormetic dose responses predominate. *Crit. Rev. Toxicol.* 38, 489–542. doi: 10.1080/10408440802014238

Calabrese, E. J. (2008c). Hormesis and medicine. *Br. J. Clin. Pharmacol.* 66, 594–617. doi: 10.1111/j.1365-2125.2008.03243.x

Calabrese, E. J. (2008d). Hormesis: why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem.* 27, 1451–1474. doi: 10.1897/07-541.1

Calabrese, E. J. (2008e). Modulation of the epileptic seizure threshold: implications of biphasic dose responses. *Crit. Rev. Toxicol.* 38, 543–556. doi: 10.1080/10408440802014261

Calabrese, E. J. (2010). Hormesis is central to toxicology, pharmacology and risk assessment. *Hum. Exp. Toxicol.* 29, 249–261. doi: 10.1177/0960327109363973

Calabrese, E. J. (2011). Toxicology rewrites its history and rethinks its future: giving equal focus to both harmful and beneficial effects. *Environ. Toxicol. Chem.* 30, 2658–2673. doi: 10.1002/etc.687

Calabrese, E. J. (2013a). Biphasic dose responses in biology, toxicology and medicine: accounting for their generalizability and quantitative features. *Environ. Pollut.* 182, 452–460. doi: 10.1016/j.envpol.2013.07.046

Calabrese, E. J. (2013b). Historical foundations of wound healing and its potential for acceleration: dose–response considerations. *Wound Repair Regen.* 21, 180–193. doi: 10.1111/j.1524-475X.2012.00842.x

Calabrese, E. J. (2013c). Hormesis: toxicological foundations and role in aging research. *Exp. Gerontol.* 48, 99–102. doi: 10.1016/j.exger.2012.02.004

Calabrese, E. J. (2013d). Hormetic mechanisms. *Crit. Rev. Toxicol.* 43, 580–606. doi: 10.3109/10408444.2013.808172

Calabrese, E. J., and Baldwin, L. A. (2000a). Chemical hormesis: its historical foundations as a biological hypothesis. *Hum. Exp. Toxicol.* 19, 2–31. doi: 10.1191/096032700678815585

Calabrese, E. J., and Baldwin, L. A. (2000b). Radiation hormesis: its historical foundations as a biological hypothesis. *Hum. Exp. Toxicol.* 19, 41–75. doi: 10.1191/096032700678815602

Calabrese, E. J., and Baldwin, L. A. (2000c). Radiation hormesis: the demise of a legitimate hypothesis. *Hum. Exp. Toxicol.* 19, 76–84. doi: 10.1191/096032700678815611

Calabrese, E. J., and Baldwin, L. A. (2000d). Tale of two similar hypotheses: the rise and fall of chemical and radiation hormesis. *Hum. Exp. Toxicol.* 19, 85–97. doi: 10.1191/096032700678815620

Calabrese, E. J., and Baldwin, L. A. (2000e). The marginalization of hormesis. *Hum. Exp. Toxicol.* 19, 32–40. doi: 10.1191/096032700678815594

Calabrese, E. J., and Baldwin, L. A. (2001). The frequency of U-shaped dose responses in the toxicological literature. *Toxicol. Sci.* 62, 330–338. doi: 10.1093/toxsci/62.2.330

Calabrese, E. J., and Baldwin, L. A. (2003). The hormetic dose–response model is more common than the threshold model in toxicology. *Toxicol. Sci.* 71, 246–250. doi: 10.1093/toxsci/71.2.246

Calabrese, E. J., and Blain, R. B. (2005). The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol. Appl. Pharmacol.* 202, 289–301. doi: 10.1016/j.taap.2004.06.023

Calabrese, E. J., and Blain, R. B. (2009). Hormesis and plant biology. *Environ. Pollut.* 157, 42–48. doi: 10.1016/j.envpol.2008.07.028

Calabrese, E. J., and Blain, R. B. (2011). The hormesis database: the occurrence of hormetic dose responses in the toxicological literature. *Regul. Toxicol. Pharmacol.* 61, 73–81. doi: 10.1016/j.yrtph.2011.06.003

Calabrese, E. J., and Calabrese, V. (2013a). Low dose radiation therapy (LD-RT) is effective in the treatment of arthritis: animal model findings. *Int. J. Radiat. Biol.* 89, 287–294. doi: 10.3109/09553002.2013.752595

Calabrese, E. J., and Calabrese, V. (2013b). Reduction of arthritic symptoms by low dose radiation therapy (LD-RT) is associated with an anti-inflammatory phenotype. *Int. J. Radiat. Biol.* 89, 278–286. doi: 10.3109/09553002.2013.752594

Calabrese, E. J., and Dhawan, G. (2013). How radiotherapy was historically used to treat pneumonia: could it be useful today? *Yale J. Biol. Med.* 86, 555–570.

Calabrese, E. J., Hoffmann, G. R., Stanek E. J. III, and Nascarella, M. A. (2010a). Hormesis in high-throughput screening of antibacterial compounds in *E. coli*. *Hum. Exp. Toxicol.* 29, 667–677. doi: 10.1177/0960327109358917

Calabrese, E. J., Mattson, M. P., and Calabrese, V. (2010b). Dose response biology: the case of resveratrol. *Hum. Exp. Toxicol.* 29, 1034–1037. doi: 10.1177/0960327110383641

Calabrese, E. J., Mattson, M. P., and Calabrese, V. (2010c). Resveratrol commonly displays hormesis: occurrence and biomedical significance. *Hum. Exp. Toxicol.* 29, 980–1015. doi: 10.1177/0960327110383625

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Calabrese, E. J., and Mattson, M. P. (2010d). Cellular stress responses, the hormesis paradigm and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. *Antioxid. Redox Signal.* 13, 1763–1811. doi: 10.1089/ars.2009.3074

Calabrese, V., Cornelius, C., Giuffrida, A. M., and Calabrese, E. J. (2010e). Cellular stress responses, mitostress and carnitine insufficiencies as critical determinants in aging and neurodegenerative disorders: role of hormesis and vitagenes. *Neurochem. Res.* 35, 1880–1915. doi: 10.1007/s11064-010-0307-z

Calabrese, V., Cornelius, C., Maiolino, L., Luca, M., Chiaramonte, R., Toscano, M. A., et al. (2010f). Oxidative stress, redox homeostasis and cellular stress response in Ménière's disease: role of vitagenes. *Neurochem. Res.* 35, 2208–2217. doi: 10.1007/s11064-010-0304-2

Calabrese, V., Cornelius, C., Mancuso, C., Lentile, R., Stella, A. M., and Butterfield, D. A. (2010g). Redox homeostasis and cellular stress response in aging and neurodegeneration. *Methods Mol. Biol.* 610, 285–308. doi: 10.1007/978-1-60327-029-8_17

Calabrese, V., Cornelius, C., Trovato, A., Cambria, M. T., Lo Cascio, M. S., Di Rienzo, L., et al. (2010h). The hormetic role of dietary antioxidants in free radical-related diseases. *Curr. Pharm. Des.* 16, 8778–8783. doi: 10.2174/138161210790883615

Calabrese, E. J., Iavicoli, I., and Calabrese, V. (2012a). Hormesis: why it is important to biogerontologists. *Biogerontology* 13, 215–235. doi: 10.1007/s10522-012-9374-7

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Iavicoli, I., Di Paola, R., Koverech, A., et al. (2012b). Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity. *Biochim. Biophys. Acta* 1822, 753–783. doi: 10.1016/j.bbadis.2011.11.002

Calabrese, E. J., Iavicoli, I., and Calabrese, V. (2013). Hormesis: its impact on medicine and health. *Hum. Exp. Toxicol.* 32, 120–152. doi: 10.1177/0960327112455069

Calabrese, E. J., and Mattson, M. P. (2011). Hormesis provides a generalized quantitative estimate of biological plasticity. *J. Cell Commun. Signal.* 5, 25–38. doi: 10.1007/s12079-011-0119-1

Calabrese, E. J., Stanek, E. J. III, Nascarella, M. A., and Hoffmann, G. R. (2008a). Hormesis predicts low-dose responses better than threshold model. *Int. J. Toxicol.* 27, 369–378. doi: 10.1080/10915810802503735

Calabrese, V., Bates, T. E., Mancuso, C., Cornelius, C., Ventimiglia, B., Cambria, M. T., et al. (2008b). Curcumin and the cellular stress response in free radical-related diseases. *Mol. Nutr. Food Res.* 52, 1062–1073. doi: 10.1002/mnfr.200700316

Calabrese, V., Butterfield, D. A., and Stella, A. M. (2008c). "Aging and oxidative stress response in the CNS," in *Development and Aging Changes in the Nervous System. Handbook of Neurochemistry and Molecular Neurobiology*, 3rd Edn, eds L. Abel, P.-P. J. Regino, and R. Steffen (New York: Springer), 128–234.

Calabrese, V., Calafato, S., Cornelius, C., Mancuso, C., and Dinkova-Kostova, A. (2008d). "Heme oxygenase: a master vitagene involved in cellular stress response," in *Enzymes and the Cellular Fight Against Oxidation*, ed. A. M. Eleuteri (Trivandrum: Research Signpost), 145–163.

Calabrese, V., Calafato, S., Puleo, E., Cornelius, C., Sapienza, M., Morganti, P., et al. (2008e). Redox regulation of cellular stress response by ferulic acid ethyl ester in human dermal fibroblasts: role of vitagenes. *Clin. Dermatol.* 26, 358–363. doi: 10.1016/j.clindermatol.2008.01.005

Calabrese, V., Cornelius, C., Mancuso, C., Ientile, R., Giuffrida Stella, A. M., and Butterfield, D. A. (2008f). "Redox homeostasis and cellular stress response in aging and neurodegeneration," in *Free Radical and Antioxidant Protocols*, 2nd Edn, eds R. M. Uppu, S. N. Murthy, W. A. Pryor, and N. L. Parinandi (New York: Humana Press), 285–308.

Calabrese, V., Cornelius, C., Mancuso, C., Pennisi, G., Calafato, S., Bellia, F., et al. (2008g). Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. *Neurochem. Res.* 33, 2444–2471. doi: 10.1007/s11064-008-9775-9

Calabrese, V., Ientile, R., Cornelius, C., Scalia, M., Cambria, M. T., Ventimiglia, B., et al. (2008h). "Nutritional redox homeostasis and cellular stress response: differential role of homocysteine and acetylcarnitine," in *Dietary Modulation of Cell Signaling Pathways*, eds Y. J. Surh, Z. Dong, E. Cadenas, and L. Packer (New York, NY: CRC Press), 229–250.

Calabrese, V., Mancuso, C., Cornelius, C., Calafato, M., Ventimiglia, B., Butterfield, D. A., et al. (2008i). "Reactive nitrogen species and cellular stress tolerance in aging and neurodegeneration: role of vitagenes," in *Free Radical Pathophysiology*, eds S. Alvarez and P. Evelson (Trivandrum: Transworld Research Network), 345–367.

Calabrese, V., Signorile, A., Cornelius, C., Mancuso, C., Scapagnini, G., Ventimiglia, B., et al. (2008j). Practical approaches to investigate redox regulation of heat shock protein expression and intracellular glutathione redox state. *Methods Enzymol.* 441, 83–110. doi: 10.1016/S0076-6879(08)01206-8

Calabrese, E. J., Staudenmayer, J. W., Stanek, E. J. III, and Hoffmann, G. R. (2006a). Hormesis outperforms threshold model in National Cancer Institute antitumor drug screening database. *Toxicol. Sci.* 94, 368–378. doi: 10.1093/toxsci/kfl098

Calabrese, V., Butterfield, D. A., Scapagnini, G., Stella, A. M., and Maines, M. D. (2006b). Redox regulation of heat shock protein expression by signaling involving nitric oxide and carbon monoxide: relevance to brain aging, neurodegenerative disorders, and longevity. *Antioxid. Redox Signal.* 8, 444–477. doi: 10.1089/ars.2006.8.444

Calabrese, V., Colombrita, C., Sultana, R., Scapagnini, G., Calvani, M., Butterfield, D. A., et al. (2006c). Redox modulation of heat shock protein expression by acetylcarnitine in aging brain: relationship to antioxidant status and mitochondrial function. *Antioxid. Redox Signal.* 8, 404–416. doi: 10.1089/ars.2006.8.404

Calabrese, V., Sultana, R., Scapagnini, G., Guagliano, E., Sapienza, M., Bella, R., et al. (2006d). Nitrosative stress, cellular stress response, and thiol homeostasis in patients with Alzheimer's disease. *Antioxid. Redox Signal.* 8, 1975–1986. doi: 10.1089/ars.2006.8.1975

Calabrese, V., Cornelius, C., Cuzzocrea, S., Iavicoli, I., Rizzarelli, E., and Calabrese, E. J. (2011). Hormesis, cellular stress response and vitagenes as critical

determinants in aging and longevity. *Mol. Aspects Med.* 32, 279–304. doi: 10.1016/j.mam.2011.10.007

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., and Calabrese, E. J. (2009a). Vitagenes, cellular stress response and acetylcarnitine: relevance to hormesis. *Biofactors* 35, 146–160. doi: 10.1002/biof.22

Calabrese, V., Cornelius, C., Mancuso, C., Barone, E., Calafato, S., Bates, T., et al. (2009b). Vitagenes, dietary antioxidants and neuroprotection in neurodegenerative diseases. *Front. Biosci.* 14:376–397. doi: 10.2741/3250

Calabrese, V., Cornelius, C., Rizzarelli, E., Owen, J. B., Dinkova-Kostova, A. T., and Butterfield, D. A. (2009c). Nitric oxide in cell survival: a Janus molecule. *Antioxid. Redox Signal.* 11, 2717–2739. doi: 10.1089/ars.2009.2721

Calabrese, V., Perluigi, M., Cornelius, C., Coccia, R., Di Domenico, F., Mancuso, C., et al. (2009d). "Phenolics in aging and neurodegenerative disorders," in *Phenolic Compounds of Plant Origin and Health: The Biochemistry behind Their Nutritional and Pharmacological Value*, ed. C. G. Fraga (New York, NY: Wiley & Sons), 427–451.

Calabrese, V., Guagliano, E., Sapienza, M., Panebianco, M., Calafato, S., Puleo, E., et al. (2007a). Redox regulation of cellular stress response in aging and neurodegenerative disorders: role of vitagenes. *Neurochem. Res.* 32, 757–773. doi: 10.1007/s11064-006-9203-y

Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D. A., and Giuffrida Stella, A. M. (2007b). Nitric oxide in the CNS: neuroprotection versus neurotoxicity. *Nat. Neurosci.* 8, 766–775. doi: 10.1038/nrn2214

Calabrese, V., Mancuso, C., Ravagna, A., Perluigi, M., Cini, C., De Marco, C., et al. (2007c). In vivo induction of heat shock proteins in the substantia nigra following L-DOPA administration is associated with increased activity of mitochondrial complex I and nitrosative stress in rats: regulation by glutathione redox state. *J. Neurochem.* 101, 709–717. doi: 10.1111/j.1471-4159.2006.04367.x

Calabrese, V., and Maines, M. D. (2006). Antiaging medicine: antioxidants and aging. *Antioxid. Redox Signal.* 8, 362–364. doi: 10.1089/ars.2006.8.362

Cervellati, C., Romani, A., Seripa, D., Cremonini, E., Bosi, C., Magon, S., et al. (2014). Systemic oxidative stress and conversion to dementia of elderly patients with mild cognitive impairment. *Biomed. Res. Int.* 2014, 309507. doi: 10.1155/2014/309507

Chico, L., Simoncini, C., Lo Gerfo, A., Rocchi, A., Petrozzi, L., Carlesi, C., et al. (2013). Oxidative stress and APO E polymorphisms in Alzheimer's disease and in mild cognitive impairment. *Free Radic. Res.* 47, 569–576. doi: 10.3109/10715762.2013.804622

Cornelius, C., Perrotta, R., Graziano, A., Calabrese, E. J., and Calabrese, V. (2013a). Stress responses, vitagenes and hormesis as critical determinants in aging and longevity: mitochondria as a "chi". *Immun. Ageing* 10:15. doi: 10.1186/1742-4933-10-15

Cornelius, C., Trovato Salinaro, A., Scuto, M., Fronte, V., Cambria, M. T., Pennisi, M., et al. (2013b). Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes. *Immun. Ageing* 10:41. doi: 10.1186/1742-4933-10-41

Cui, S., Xiong, F., Hong, Y., Jung, J. U., Li, X. S., Liu, J. Z., et al. (2011). APPswe/Aβ regulation of osteoclast activation and RAGE expression in an age-dependent manner. *J. Bone Miner. Res.* 26, 1084–1098. doi: 10.1002/jbmr.299

Dawkins, E., and Small, D. H. (2014). Insights into the physiological function of the β-amyloid precursor protein: beyond Alzheimer's disease. *J. Neurochem.* doi: 10.1111/jnc.12675 [Epub ahead of print].

Di Domenico, F., Perluigi, M., Butterfield, D. A., Cornelius, C., and Calabrese, V. (2010). Oxidative damage in rat brain during aging: interplay between energy and metabolic key target proteins. *Neurochem. Res.* 35, 2184–2192. doi: 10.1007/s11064-010-0295-z

Di Paola, R., Impellizzeri, D., Trovato Salinaro, A., Mazzon, E., Bellia, F., Cavallaro, M., et al. (2011). Administration of carnosine in the treatment of acute spinal cord injury. *Biochem. Pharm.* 82, 1478–1489. doi: 10.1016/j.bcp.2011.07.074

Edrey, Y. H., Oddo, S., Cornelius, C., Caccamo, A., Calabrese, V., and Buffenstein, R. (2014). Oxidative damage and amyloid-β metabolism in brain regions of the longest-lived rodents. *J. Neurosci. Res.* 92, 195–205. doi: 10.1002/jnr.23320

Fang, C. L., Wu, W. H., Liu, Q., Sun, X., Ma, Y., Zhao, Y. F., et al. (2010). Dual functions of beta-amyloid oligomer and fibril in Cu(II)-induced H_2O_2 production. *Regul. Pept.* 163, 1–6. doi: 10.1016/j.regpep.2010.05.001

Fujimoto, M., Hayashida, N., Katoh, T., Oshima, K., Shinkawa, T., Prakasam, R., et al. (2010). A novel mouse HSF3 has the potential to activate nonclassical heat-shock genes during heat shock. *Mol. Biol. Cell* 21, 106–116. doi: 10.1091/mbc.E09-07-0639

Gupta, S. C., Kim, J. H., Kannappan, R., Reuter, S., Dougherty, P. M., and Aggarwal, B. B. (2011). Role of nuclear factor kappaB-mediated inflammatory pathways in cancer-related symptoms and their regulation by nutritional agents. *Exp. Biol. Med. (Maywood)* 236, 658–671. doi: 10.1258/ebm.2011.011028

Hardas, S. S., Sultana, R., Clark, A. M., Beckett, T. L., Szweda, L. I., Murphy, M. P., et al. (2013). Oxidative modification of lipoic acid by HNE in Alzheimer disease brain. *Redox Biol.* 1, 80–85. doi: 10.1016/j.redox.2013.01.002

Hong, L., Huang, H. C., and Jiang, Z. F. (2014). Relationship between amyloid-beta and the ubiquitin-proteasome system in Alzheimer's disease. *Neurol. Res.* 36, 276–282. doi: 10.1179/1743132813Y.0000000288

Hughes, T. M., Lopez, O. L., Evans, R. W., Kamboh, M. I., Williamson, J. D., Klunk, W. E., et al. (2014). Markers of cholesterol transport are associated with amyloid deposition in the brain. *Neurobiol. Aging* 35, 802–807. doi: 10.1016/j.neurobiolaging.2013.09.040

Jefferies, W. A., Price, K. A., Biron, K. E., Fenninger, F., Pfeifer, C. G., and Dickstein, D. L. (2013). Adjusting the compass: new insights into the role of angiogenesis in Alzheimer's disease. *Alzheimers Res. Ther.* 5:64. doi: 10.1186/alzrt230

Jiang, J., Wang, Y., Hou, L., Fan, L., Wang, Q., Xu, Z., et al. (2013). Distinct roles of sAPP-α and sAPP-β in regulating U251 cell differentiation. *Curr. Alzheimer Res.* 10, 706–713. doi: 10.2174/15672050113109990141

Kang, J. H., Korecka, M., Toledo, J. B., Trojanowski, J. Q., and Shaw, L. M. (2013). Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid-β(1-42) and τ-proteins as Alzheimer disease biomarkers. *Clin. Chem.* 59, 903–916. doi: 10.1373/clinchem.2013.202937

Kanzaki, H., Shinohara, F., Kajiya, M., and Kodama, T. (2013). The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. *J. Biol. Chem.* 288, 23009–230020. doi: 10.1074/jbc.M113.478545

Kaunitz, J. D., and Yamaguchi, D. T. (2008). TNAP, TrAP, ecto-purinergic signaling, and bone remodeling. *J. Cell. Biochem.* 105, 655–662. doi: 10.1002/jcb.21885

Krenz, M., Baines, C., Kalogeris, T., and Korthuis, R. J. (2013). "Cell survival programs and ischemia/reperfusion: hormesis, preconditioning, and cardioprotection," in *Colloquium Series on Integrated Systems Physiology: From Molecule to Function to Disease*, Vol. 109, eds D. N. Granger and J. Granger (San Rafael, CA: Morgan & Claypool Life Science Digital Library), 1–32.

Li, S., Liu, B., Zhang, L., and Rong, L. (2014). Amyloid beta peptide is elevated in osteoporotic bone tissues and enhances osteoclast function. *Bone* 61C, 164–175. doi: 10.1016/j.bone.2014.01.010

Liu, L., and Chan, C. (2014). The role of inflammasome in Alzheimer's disease. *Ageing Res. Rev.* 15, 6–15. doi: 10.1016/j.arr.2013.12.007

Lodi, R., Tonon, C., Calabrese, V., and Schapira, A. H. (2006). Friedreich's ataxia: from disease mechanisms to therapeutic interventions. *Antioxid. Redox Signal.* 8, 438–443. doi: 10.1089/ars.2006.8.438

Mancuso, C., Bates, T. E., Butterfield, D. A., Calafato, S., Cornelius, C., De Lorenzo, A., et al. (2007a). Natural antioxidants in Alzheimer's disease. *Expert Opin. Investig. Drugs* 16, 1921–1931. doi: 10.1517/13543784.16.12.1921

Mancuso, C., Scapagnini, G., Curro, D., Giuffrida Stella, A. M., De Marco, C., Butterfield, D. A., et al. (2007b). Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. *Front. Biosci.* 12:1107–1123. doi: 10.2741/2130

Mancuso, C., Capone, C., Ranieri, S. C., Fusco, S., Calabrese, V., Eboli, M. L., et al. (2008). Bilirubin as an endogenous modulator of neurotrophin redox signaling. *J. Neurosci. Res.* 86, 1212–1230. doi: 10.1002/jnr.21665

Mancuso, C., Pani, G., and Calabrese, V. (2006). Bilirubin: an endogenous scavenger of nitric oxide and reactive nitrogen species. *Redox Rep.* 11, 207–213. doi: 10.1179/135100006X154978

Mizoi, M., Yoshida, M., Saiki, R., Waragai, M., Uemura, K., Akatsu, H., et al. (2014). Distinction between mild cognitive impairment and Alzheimer's disease by CSF amyloid β(40) and β(42), and protein-conjugated acrolein. *Clin. Chim. Acta.* 430C, 150–155. doi: 10.1016/j.cca.2014.01.007

Murshid, A., Eguchi, T., and Calderwood, S. K. (2013). Stress proteins in aging and life span. *Int. J. Hyperthermia* 29, 442–447. doi: 10.3109/02656736.2013.798873

Ono, K., Ohashi, S., and Tanaka, S. (2014). New Diagnostic Criteria and Guidelines on Osteoporosis. Anti-osteoporosis drugs based on the guidelines for the Prevention and Treatment of Osteoporosis (2011 edition). *Clin. Calcium* 24, 401–406. doi: CliCa1403401406

Osorio, R. S., Pirraglia, E., Gumb, T., Mantua, J., Ayappa, I., Williams, S., et al. (2014). Imaging and cerebrospinal fluid biomarkers in the search for Alzheimer's disease mechanisms. *Neurodegener. Dis.* 13, 163–165. doi: 10.1159/000355063

Park, K., Ju, W. C., Yeo, J. H., Kim, J. Y., Seo, H. S., Uchida, Y., et al. (2014). Increased OPG/RANKL ratio in the conditioned medium of soybean-treated osteoblasts suppresses RANKL-induced osteoclast differentiation. *Int. J. Mol. Med.* 33, 178–184. doi: 10.3892/ijmm.2013.1557

Park, S. K., Oh, S., Shin, H. K., Kim, S. H., Ham, J., Song, J. S., et al. (2011). Synthesis of substituted triazolyl curcumin mimics that inhibit RANKL-induced osteoclastogenesis. *Bioorg. Med. Chem. Lett.* 21, 3573–3577. doi: 10.1016/j.bmcl.2011.04.106

Pennisi, G., Cornelius, C., Cavallaro, M. M., Trovato Salinaro, A., Cambria, M. T., Pennisi, M., et al. (2011). Redox regulation of cellular stress response in multiple sclerosis. *Biochem. Pharm.* 82, 1490–1499. doi: 10.1016/j.bcp.2011.07.092

Perluigi, M., Di Domenico, F., Giorgi, A., Schininà, M. E., Coccia, R., Cini, C., et al. (2010). Redox proteomics in aging rat brain: involvement of mitochondrial reduced glutathione status and mitochondrial protein oxidation in the aging process. *J. Neurosci. Res.* 88, 3498–3507. doi: 10.1002/jnr.22500

Perluigi, M., Joshi, G., Sultana, R., Calabrese, V., De Marco, C., Coccia, R., et al. (2006). In vivo protective effects of ferulic acid ethyl ester against amyloid-beta peptide 1-42-induced oxidative stress. *J. Neurosci. Res.* 84, 418–426. doi: 10.1002/jnr.20879

Piva, R., Penolazzi, L., Borgatti, M., Lampronti, I., Lambertini, E., Torreggiani, E., et al. (2009). Apoptosis of human primary osteoclasts treated with molecules targeting nuclear factor-kappaB. *Ann. N. Y. Acad. Sci.* 1171, 448–456. doi: 10.1111/j.1749-6632.2009.04906.x

Ponnayyan Sulochana, S., Sharma, K., Mullangi, R., and Sukumaran, S. K. (2014). Review of the validated HPLC and LC-MS/MS methods for determination of drugs used in clinical practice for Alzheimer's disease. *Biomed. Chromatogr.* doi: 10.1002/bmc.3116. [Epub ahead of print].

Rachner, T. D., Khosla, S., and Hofbauer, L. C. (2011). Osteoporosis: now and the future. *Lancet* 377, 1276–1287. doi: 10.1016/S0140-6736(10)62349-5

Richard, E., and Brayne, C. (2014). Dementia: mild cognitive impairment – not always what it seems. *Nat. Rev. Neurol.* 10, 130–131. doi: 10.1038/nrneurol.2014.23

Roher, A. E., Esh, C. L., Kokjohn, T. A., Castano, E. M., Van Vickle, G. D., Kalback, W. M., et al. (2009). Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimers Dement.* 5, 18–29. doi: 10.1016/j.jalz.2008.10.004

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nat. Rev. Mol. Cell Biol.* 14, 630–642. doi: 10.1038/nrm3658

Scapagnini, G., Caruso, C., and Calabrese, V. (2011). Therapeutic potential of dietary polyphenols against brain ageing and neurodegenerative disorders. *Adv. Exp. Med. Biol.* 698, 27–35. doi: 10.1007/978-1-4419-7347-4_3

Scapagnini, G., Colombrita, C., Amadio, M., D'Agata, V., Arcelli, E., Sapienza, M., et al. (2006). Curcumin activates defensive genes and protects neurons against oxidative stress. *Antioxid. Redox Signal.* 8, 395–403. doi: 10.1089/ars.2006.8.395

Schulz, H. (1887). Zur Lehre von der Arzneiwirdung. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* 108, 423–445. doi: 10.1007/BF02281473

Schulz, H. (1888). Uber Hefegifte. *Pflugers Arch. Physiol. Menschen Tiere* 42, 517–541. doi: 10.1007/BF01669373

Siciliano, R., Barone, E., Calabrese, V., Rispoli, V., Butterfield, D. A., and Mancuso, C. (2011). Experimental research on nitric oxide and the therapy of Alzheimer disease: a challenging bridge. *CNS Neurol. Disord. Drug Targets* 10, 766–776. doi: 10.2174/187152711798072356

Southam, C. M., and Ehrlich, J. (1943). Effects of extract of western red-cedar heartwood on certain wood-decaying fungi in culture. *Phytopathology* 33, 517–524.

Soysa, N. S., Alles, N., Weih, D., Lovas, A., Mian, A. H., Shimokawa, H., et al. (2010). The pivotal role of the alternative NF-kappaB pathway in maintenance of basal bone homeostasis and osteoclastogenesis. *J. Bone Miner. Res.* 25, 809–818. doi: 10.1359/jbmr.091030

Stefanova, N. A., Kozhevnikova, O. S., Vitovtov, A. O., Maksimova, K. Y., Logvinov, S. V., Rudnitskaya, E. A., et al. (2014). Senescence-accelerated OXYS rats: a model of age-related cognitive decline with relevance to abnormalities in Alzheimer disease. *Cell Cycle* 13, 898–909. doi: 10.4161/cc.28255

Sun, S. C. (2012). The noncanonical NF-κB pathway. *Immunol. Rev.* 246, 125–140. doi: 10.1111/j.1600-065X.2011.01088.x

Swomley, A. M., Förster, S., Keeney, J. T., Triplett, J., Zhang, Z., Sultana, R., et al. (2013). Abeta, oxidative stress in Alzheimer disease: evidence based on proteomics studies. *Biochim. Biophys. Acta* doi: 10.1016/j.bbadis.2013.09.015 [Epub ahead of print].

Tóth, G., Gardai, S. J., Zago, W., Bertoncini, C. W., Cremades, N., Roy, S. L., et al. (2014). Targeting the intrinsically disordered structural ensemble of α-synuclein by small molecules as a potential therapeutic strategy for Parkinson's disease. *PLoS ONE* 9:e87133. doi: 10.1371/journal.pone.0087133

Vacek, T. P., Kalani, A., Voor, M. J., Tyagi, S. C., and Tyagi, N. (2013). The role of homocysteine in bone remodeling. *Clin. Chem. Lab. Med.* 51, 579–590. doi: 10.1515/cclm-2012-0605

Valasani, K. R., Sun, Q., Hu, G., Li, J., Du, F., Guo, Y., et al. (2014). Identification of human ABAD inhibitors for rescuing Aβ-mediated mitochondrial dysfunction. *Curr. Alzheimer Res.* 11, 128–136. doi: 10.2174/1567205011666140130150108

Wallace, D. C. (2008). Mitochondria as chi. *Genetics* 179, 727–735. doi: 10.1534/genetics.104.91769

Wallace, D. C. (2010). Mitochondrial DNA mutations in disease and aging. *Environ. Mol. Mutagen.* 5, 440–450. doi: 10.1002/em.20586

Wallace, D. C. (2012). Mitochondria and cancer. *Nat. Rev. Cancer* 10, 685–698. doi: 10.1038/nrc3365

Wallace, D. C. (2013). Bioenergetics in human evolution and disease: implications for the origins of biological complexity and the missing genetic variation of common diseases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368, 20120267. doi: 10.1098/rstb.2012.0267

Westerheide, S. D., Raynes, R., Powell, C., Xue, B., and Uversky, V. N. (2012). HSF transcription factor family, heat shock response, and protein intrinsic disorder. *Curr. Protein Pept. Sci.* 13, 86–103. doi: 10.2174/138920312799277956

Willcox, B. J., and Willcox, D. C. (2014). Caloric restriction, caloric restriction mimetics, and healthy aging in Okinawa: controversies and clinical implications. *Curr. Opin. Clin. Nutr. Metab. Care* 17, 51–58. doi: 10.1097/MCO.0000000000000019

Woodman, I. (2013). Osteoporosis: linking osteoporosis with Alzheimer disease. *Nat. Rev. Rheumatol.* 9, 638. doi: 10.1038/nrrheum.2013.152

Xia, W. F., Jung, J. U., Shun, C., Xiong, S., Xiong, L., Shi, X. M., et al. (2013). Swedish mutant APP suppresses osteoblast differentiation and causes osteoporotic deficit, which are ameliorated by *N*-acetyl-L-cysteine. *J. Bone Miner. Res.* 28, 2122–2135. doi: 10.1002/jbmr.1954

Zhang, Y., Ahn, Y. H., Benjamin, I. J., Honda, T., Hicks, R. J., Calabrese, V., et al. (2011). HSF1-dependent upregulation of Hsp70 by sulfhydryl-reactive inducers of the KEAP1/NRF2/ARE pathway. *Chem. Biol.* 18, 1355–1361. doi: 10.1016/j.chembiol.2011.09.008

Zhang, Y., Naidu, D. S., Kostov, R. V., Pheely, A., Calabrese, V., and Dinkova-Kostova, A. T. (2014). "Sulfhydryl-reactive phytochemicals as dual activators of transcription factors NRF2 and HSF1," in *Recent Advances in Phytochemistry, 50 Years of Phytochemistry Research*, ed. D. R. Gang (New York, NY: Springer), 95–119.

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Nutraceuticals in cognitive impairment and Alzheimer's disease

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Several chemical substances belonging to classes of natural dietary origin display protective properties against some age-related diseases including neurodegenerative ones, particularly Alzheimer's disease (AD). These compounds, known as nutraceuticals, differ structurally, act therefore at different biochemical and metabolic levels and have shown different types of neuroprotective properties. The aim of this review is to summarize data from observational studies, clinical trials, and randomized clinical trials (RCTs) in humans on the effects of selected nutraceuticals against age-related cognitive impairment and dementia. We report results from studies on flavonoids, some vitamins and other natural substances that have been studied in AD and that might be beneficial for the maintenance of a good cognitive performance. Due to the substantial lack of high-level evidence studies there is no possibility for recommendation of nutraceuticals in dementia-related therapeutic guidelines. Nevertheless, the strong potential for their neuroprotective action warrants further studies in the field.

Keywords: cognitive impairment, dementia, Alzheimer's disease, dietary natural substances, neurodegeneration, nutraceuticals, neuroprotection

INTRODUCTION

Advanced age is often characterized by a decline in a large spectrum of cognitive abilities including reasoning, memory, perceptual speed, and language. The impairment of more of these activities, when lasting long enough and being associated to functional loss is referred to as dementia. Alzheimer's disease (AD) is the most common and feared form of dementia representing circa 70% of all dementia cases and displaying a dramatic epidemics due to the enormous growth of the aged population worldwide. Incidence of dementia has increased over the recent years although recent epidemiological studies seem to show a decline that needs to be confirmed in the future (Larson et al., 2013). The number of cases is expected to approach a million people per year in USA by 2050 (Alzheimer's Association, 2009). AD impacts dramatically on everyday life of older adults, being one of the main causes of disability in the old age. There has been an increasing interest in the past decades about interventions that may help to improve cognitive performance in older age or, at least, delay the onset of dementia. Due to the absence of a cure against dementia and AD, the public health priority has focused more recently on prevention of cognitive decline. It is still unclear which factors lead to the molecular cascade of neurodegeneration in AD, but along with genetic and environmental factors vascular pathology and risk factors have been recently shown to play a crucial role in AD pathogenesis (Polidori et al., 2012). Therefore, lifestyle strategies with beneficial effects on neurodegeneration and vascularity – including natural nutrition and nutritional supplementation, cognitive and social activity, physical exercise – have been identified as possible target options for AD prevention (Brown et al., 2010; Polidori et al., 2012; Polidori and Schulz, 2014).

The effect of a correct diet on human health has been reported in many epidemiological studies and randomized controlled trials (RCTs; Everitt et al., 2006). There is clear evidence that a diet rich in specific nutritional food groups (fruit, fish, vegetables) can reduce the incidence and prevalence of some of the main clinical outcomes, such as neurodegenerative disorders, cardiovascular diseases, diabetes, cancer (Grant, 1999; Panza et al., 2004; Sofi et al., 2008; Frisardi et al., 2010). These specific nutritional food groups are rich in micronutrients and vitamins that, for their characteristic of being of nutritional nature and beneficial for health (like pharmaceuticals) at the same time, have been defined as *nutraceuticals* by De Felice in 1989 (Kalra, 2003). Among different types of diet, the Mediterranean pattern obtained a considerable amount of interest in the past recent decades due to the results of large epidemiological and bench studies showing its high content in nutraceuticals. The Mediterranean diet is characterized by a high consumption of plant foods, fish, olive oil as primary sources of monounsaturated fat and moderate intake of wine. This kind of food intake pattern might be particularly healthy due to synergistic actions of its components. Synergistic mechanisms between food components are responsible particularly for the neuroprotective effects displayed by certain nutrients and nutraceuticals. In addition, cardiovascular diseases such as diabetes mellitus, hypertension, and lipid disorders, as well as white substance lesions are highly susceptible to micronutrient changes. This might be particularly important in light of the recently shown major pathophysiological role of vascular pathology and risk factors in both AD and its prodromal phase, mild cognitive impairment (MCI; De la Torre, 2010; Polidori et al., 2012; Féart et al., 2013). Independent of the mechanism through which the Mediterranean dietary pattern exerts its

beneficial effects, a large amount of studies have recently shown its protective activity against MCI and AD (systematically reviewed and meta-analyzed by Singh et al., 2013 and Psaltopoulou et al., 2013).

The aim of this review was to present evidence on a particular group of plant and food components, the so-called nutraceuticals, which have displayed over the years the ability or a strong potential to act as neuroprotectans and/or delay cognitive impairment.

Practically, no official definition exists for the term "nutraceutical," though it is often used to describe a broad list of products sold under the premise of food components with an expressed intent of treatment or prevention of disease and for enhancing the health and wellbeing of an individual (Hardy et al., 2003). In other words, nutraceuticals are foods, or food components, that provide medical or health benefits, including prevention and treatment of several diseases (Calabrese et al., 2009).

In this review, we focused our attention on a group of substances proposed to prevent or treat cognitive impairment or dementia, with a particular attention on AD. Nutraceuticals evaluated in humans in epidemiological, observational, or clinical studies as well as in RCTs were selected. Only molecules with stronger evidence for neuroprotection as well as those more widely studied were included. To avoid redundancies, we refer the reader to our previous studies for the role and use of vitamin E family (Mangialasche et al., 2010, 2012, 2013; Mecocci and Polidori, 2012; Mecocci et al., 2013) and to recent reviews for the role and use of vitamin C (Heo et al., 2013; Harrison et al., 2014) and of omega-3 fatty acids (Lin et al., 2012; Sydenham et al., 2012; Dacks et al., 2013; Denis et al., 2013).

FLAVONOIDS

Flavonoids are a group of poliphenolic compounds that are very common in the daily human diet. They are found in most plants, including fruits, vegetables, and several types of natural drinks such as tea, cocoa and wine (Manach et al., 2004; Spencer et al., 2008).

On the basis of their chemical structure they can be divided into six subgroups (**Table 1**).

Flavonoids and their metabolites modulate several neurological processes as shown by studies in which an interaction with neuronal-glial signaling pathways involved in neuronal survival and function was observed (Spencer, 2010; Williams and Spencer, 2012). In addition, flavonoids induce changes in cerebral blood flow (Francis et al., 2006; Fisher et al., 2006; Williams and Spencer, 2012), upregulate antioxidant enzymes and proteins involved in synaptic plasticity and neuronal repair (Mann et al., 2007; Eggler et al., 2008; Spencer, 2009) and inhibit neuropathological processes in brain regions typically involved in AD pathogenesis (Wang et al., 2008a).

FLAVANOLS: CATECHIN, EPICATECHIN, EPIGALLOCATECHIN, EPIGALLOCATHECHIN GALLATE

Flavanols are a main flavonoid group and are found in cocoa and chocolate as well as in black and green tea and in grapes. Research over the past decade has identified flavanols as showing diverse beneficial physiologic and antioxidant effects, particularly in the context of vascular function (Francis et al., 2006).

Catechin and epicatechin are the most abundant flavanols in grape seeds and grape juice. A study on supplementation of grape juice from a variety of *Vitis vinifera* called Koshu demonstrated an inhibition of glutamate excitotoxicity (Narita et al., 2011). In humans, few clinical trials with grape juice have shown that short- and moderate-term supplementation produces benefit in individuals with cerebrovascular diseases including increased serum antioxidant capacity and reduced LDL oxidation, improvement of endothelial function and reduction of platelet aggregation (Krikorian et al., 2010a).

Daily EGCG treatment in rats has been demonstrated to reduce the progressive increase of oxidative stress induced by hypertension. EGCG also decreases the concentration of reactive oxygen species on hippocampus. Based on these results a therapeutic effect of EGCG in treating vascular-induced learning and memory impairment has been proposed (Wang et al., 2012). Black and green teas have a high content of catechins, being EGCG the most abundant. There are several studies that show the protective effect of catechins in cellular and animal models of AD. Despite the lack of clinical trials with tea polyphenols in neurodegenerative diseases, epidemiological observations in US and Finnish populations showed a reduced risk of Parkinson's disease in high consumers of tea (Checkoway et al., 2002; Hu et al., 2007) and a reduced risk of cognitive impairment in a Japanese population drinking green tea (Kuriyama et al., 2006).

Consumption of cocoa flavanols has been previously shown to influence cerebral hemodynamics. It has been suggested that one consequence of the effect of cocoa flavanols on cerebral blood flow

Table 1 | Flavonoid chemical subgroups and relative food sources.

Groups	Molecules	Food source
FLAVANOLS	Catechin, epicatechin, epigallocathechin, epigallocatechin gallate (EGCG)	Cocoa and chocolate, green tea, grapes
FLAVONOLS	Kaempferol, quercetin	Onions, apples, green tea, capers, leeks, broccoli
FLAVONES	Luteolin, apigenin	Celery, parsley, rosemary
ISOFLAVONES	Daidzein, genistein	Soy
FLAVANONES	Hesperetin, naringenin	Citrus fruit, tomatoes
ANTHOCYANIDINS	Pelargonidin, cyanidine, malvidin	Berry fruits, red wine

might be to improve performance on visual and cognitive tasks as shown after drinking of cocoa beverages (Scholey et al., 2010; Field et al., 2011).

FLAVONOLS: QUERCETIN, KAEMPFEROL

Quercetin, one of the most studied bioflavonoids, is found in many common foods, such as capers, apples, onions and green tea (Kelsey et al., 2010). Its primary activity is to prevent endothelial apoptosis caused by oxidants, thanks to a highly potent antioxidant activity and cytoprotective effects (Dong et al., 2012). There are several *in vitro* studies about quercetin effects demonstrating that this molecule increases cell survival in neurotoxic conditions as in the presence of hydrogen peroxide (Heo and Lee, 2004), linoleic acid hydroperoxide (Sasaki et al., 2003; Kelsey et al., 2010), interleukin-1β (Sharma et al., 2007; Kelsey et al., 2010). *In vivo* studies have demonstrated that quercetin could have a role in vascular dementia by decreasing the size of ischemic lesions (Dajas et al., 2003; Kelsey et al., 2010). Quercetin improves memory and hippocampal synaptic plasticity in models of impairment induced by chronic lead exposure and it could have a role in neuronal repairing, as shown in spinal cord injury models (Schültke et al., 2003).

Kaempferol is widely distributed in the human daily diet such as fruits, beverages, tea, and vegetables (Aherne and O'Brien, 2002). Kaempferol protects PC12 cells against the oxidative stress induced by H_2O_2 (Hong et al., 2009) and improves cognitive learning and memory capability in mice (Lei et al., 2012). It was reported that the intake of flavonols including quercetin, kaempferol, and myricetin has favorable effects on cognitive performance (Spencer, 2009).

Ginkgo biloba (Gb) is a plant whose herbal extracts (mainly EGb761) are often used as an alternative treatment to improve cognitive function. Extracts of Gb include several components, such as the flavonols quercetin and kaempferol as well as terpenoid lactones that are considered to be responsible for the neuroprotective functions of Gb (Rendeiro et al., 2012). Standardized extracts of Gb leaves are studied for their potential to improve memory and cognitive function in general. Hemodynamic, neurotransmitter, and free-radical scavenging effects of Gb have been shown in several studies. All of these biological functions may be relevant to aging and age-related disorders (Maclennan et al., 2002; Brown et al., 2010). For this reason, several *in vivo* studies from humans have been performed and beneficial effects of Gb in prevention and treatment of neurodegenerative disorders like AD have been shown. Improvement of cognitive performance (Le Bars et al., 1997; Kanowski and Hoerr, 2003), memory (Kanowski and Hoerr, 2003), and attention (Le Bars, 2003; Chan et al., 2007) were consistently observed. Two large RCTs on the use of Gb extracts (the GEM and the GuidAge study) did not show less cognitive decline over time in older adults with normal cognition or MCI taking Gb than those assuming placebo (Snitz et al., 2009; Vellas et al., 2012). Also, Gb showed no effects in reducing either the overall incidence rate of dementia or AD in old age individuals with normal cognition or MCI (De Kosky et al., 2008). The latest published Cochrane review including 36 RCTs could not report a significant evidence for a predictable clinical benefit of Gb for people with dementia or cognitive impairment (Birks and Grimley Evans, 2009).

FLAVONES: LUTEOLIN, APIGENIN

Luteolin is the most abundant flavonoid in plants such as rosemary, celery, and parsley (Chowdhury et al., 2002; El Omri et al., 2012). It has been shown that luteolin has several pharmacological properties including a protective role of DNA against hydrogen peroxide-induced toxicity and anti-inflammatory actions (Cheng et al., 2010; El Omri et al., 2012).

Apigenin was shown to protect neurons against Aβ-mediated toxicity induced by copper, to increase neuronal viability as well as to relieve mitochondrial membrane dissipation and neuronal nuclear condensation (Zhao et al., 2013). Apigenin also modulates GABAergic and glutamatergic transmission in cultured cortical neurons (Losi et al., 2004). Up to now no clinical studies have been performed with luteolin and apigenin in cognitive impairment or AD.

ISOFLAVONES: SOY - GENISTEIN, DAIDZEIN, GLYCITIN

Soybean (soy) is a rich source of phytoestrogens, especially isoflavones. These are not the only constituents of soy; in fact, soybean contains also several minerals, fibers, proteins and oligosaccharides. The isoflavones from soybean are considered agonists of estrogen receptors. The presence of soy isoflavones may be responsible for the observed memory-improving effects of soybean supplementation. Isoflavones appear to improve cognitive function by mimicking the effects of estrogen, in particular through estrogen receptor β in the brain (Lee et al., 2004a). Estrogen replacement can improve cholinergic function by increasing choline uptake and potassium-stimulated acetylcholine release (Gabor et al., 2003; Bansal and Parle, 2010). Former studies revealed that soy isoflavones improve visual spatial memory and learning ability as well as memory of male and female animals (Duncan et al., 2003; Lee et al., 2004b) and humans (File et al., 2001; Zhao and Brinton, 2007; Bansal and Parle, 2010). Furthermore, soy isoflavones can influence the brain cholinergic system and reduce age-related neuronal loss and cognition decline in male rats (Lee et al., 2004b). A study on young and mature mice (Bansal and Parle, 2010) demonstrated that chronic dietary supplementation with soybean improves cognitive performance, decreases thiobarbituric acid reactive substances (TBARs) and increases plasma glutathione peroxidase levels, suggesting that soy isoflavones have antioxidant properties (Djuric et al., 2001; Lee et al., 2004b).

Few RCTs have been performed with soy supplementation, with controversial results. A long-term supplementation of soy in women had no effect on global cognition but improved visual memory after thirty months (Henderson et al., 2012), while in men, treated for twelve weeks, only spatial working memory improved compared to the placebo group (Thorp et al., 2009). A previous study in postmenopausal women who received soy protein for twelve months had no benefit in cognitive performance (Kreijkamp-Kaspers et al., 2004).

ANTHOCYANIDINS: PELARGONIDIN, CYANIDINE, MALVIDIN

Blueberry, bilberry, cranberry, elderberry, raspberry seeds, and strawberry are sources of natural anthocyanin antioxidants. Proanthocyanidins extracted from grape seeds (the bark of the Chinese *Scutellaria baicalensis* herb) exert potent

anti-inflammatory, antioxidant, antinociceptive, and vasodilatative effects and may show antidepressant properties (Ogle et al., 2013). Berry anthocyanins also improve neuronal and cognitive brain function, ocular health as well as protect genomic DNA integrity (Zafra-Stone et al., 2007). However, blueberries also contain significant amounts of flavanols, flavonols, and other phenolics which may justify their role in increasing their beneficial effects (Harnly et al., 2006).

After blueberries feeding, anthocyanidins are found in specific cerebral sites, including hippocampus and neocortex (Andres-Lacueva et al., 2005). Neurogenesis acting on hippocampus may represent one mechanism by which blueberry flavonoids improve memory. There is strong evidence suggesting that blueberry can improve memory and learning in aged animals. These improvements seem linked to the modulation of important structural and synaptic plasticity markers (Rendeiro et al., 2012). One of the role of anthocyanins in neuroprotection could be mediated through phospholipase A2 inhibition (Frisardi et al., 2010), which is negatively involved in a complex network of signaling pathways linking receptor agonists, oxidants, and proinflammatory cytokines to the release of arachidonic acid and eicosanoid synthesis (Sun et al., 2004).

Memory performance has been demonstrated to be linked to the modulation of the expression of particular proteins like CREB (cAMP-response element-binding protein), which is a pathway known to be activated in response to Aβ and brain-derived neurotrophic factor (BDNF). Changes in CREB and BDNF in berry-feed animals were accompanied by increases in the phosphorylation state of the protein factor ERK, very important for synaptic plasticity and memory formation (Williams et al., 2008). Furthermore, blueberry seems to have a more significant effect on short-term memory than long-term memory, as demonstrated by improved performance in several memory maze tasks (Ramirez et al., 2005; Williams et al., 2008; Rendeiro et al., 2012). Another study (Fuentealba et al., 2011) that underlines the role of berries-extract against Aβ shows how these extracts could partially antagonize two newly found effects of Aβ: the decrease in intracellular Ca2+ activity, an important element in neurodegenerative processes and ATP leakage, an effect of aggregated Aβ (Petrozzi et al., 2007). Short-time blueberry diet might produce benefits on memory in aged rats (Malin et al., 2011) by a suggested alteration of ROS signaling through CREB and MAP-kinase (Brewer et al., 2010). Inflammation pathways and modulation of the expression of inflammatory genes might also be involved (Shukitt-Hale et al., 2008). Finally, there is evidence that anthocyanins have insulin-like and glitazone-like properties which may contribute to improve metabolic function and lipid lowering effects (Kalt et al., 2008; Tsuda, 2008; Krikorian et al., 2010b) as well as to improve memory and reduce depressive symptoms (Krikorian et al., 2010b).

In humans, a prospective evaluation with a food frequency questionnaire showed that a greater intake of blueberries and strawberries is associated with slower rates of cognitive decline in subjects older than seventy years, suggesting the potential protective role of berries on different cognitive functions (Devore et al., 2012).

NON-FLAVONOID POLYPHENOLS: RESVERATROL AND CURCUMIN

There is evidence that both resveratrol and curcumin, non-flavonoid polyphenols, show beneficial effects in cell culture and *in vivo* models of neurotoxicity and neurodegeneration.

Resveratrol is a polyphenol found enriched in seeds and skin of several fruits, including grapes used for red wine. It is well known mostly for its cardiovascular effects (Bertelli and Das, 2009; Kelsey et al., 2010). Recent evidence has shown that resveratrol can increase 5-HT activity, which could explain its antidepressant properties (Ogle et al., 2013). In animal models it has been shown that resveratrol inhibits noradrenalin and 5-HT reuptake in rats, with increasing hippocampal serotonin (Xu et al., 2010). Resveratrol is also able to reduce inflammation and protect neurons from death, as shown by *in vivo* experiments on animal models of oxidation-induced neuronal toxicity (Alvira et al., 2007; Kelsey et al., 2010). Protection of organotypic hippocampal slices from hydroperoxide insults has been also observed (Karlsson et al., 2000; Kelsey et al., 2010). Other possible mechanisms for the neuroprotective action displayed by resveratrol are related to its antioxidant properties and its capacity of modulation of Aβ processing and up-regulation of the longevity-linked gene sirtuin 1 (Pocernich et al., 2011).

Several studies in humans have shown a lower risk of dementia in subjects drinking moderate amounts of red wine when compared to abstainers (Orgogozo et al., 1997; Truelsen et al., 2002). Furthermore, a small clinical trial in healthy adults showed an increase of cerebral blood flow during cognitive tasks in subjects treated with resveratrol compared to placebo (Kennedy et al., 2010).

Curcumin is the most active element of tumeric (*Curcuma longa*), an herb of the ginger family. It has been a staple of oriental medicine for thousands of years (Ogle et al., 2013). Since the prevalence of AD in Indian countries is much lower than in US, it has been suggested that a diet rich in curcumin may reduce the risk of AD (Ganguli et al., 2000). Curcumin seems to act with different mechanisms including antioxidant, anti-inflammatory and anti-amylodogenic ones. For example, curcumin enhances cell viability by decreasing ROS and inhibiting pro-apoptotic signals in mouse models of encephalitis (Dutta et al., 2009; Kelsey et al., 2010). It also reduces Aβ-related cerebral burden and inflammation in transgenic AD mice (Lim et al., 2001). It has been proposed that curcumin exerts most of its effects by inhibiting monoamine oxidase levels, thereby reducing depression also because it modulates serotonergic, dopaminergic, and noradrenergic transmission (Xu et al., 2012; Ogle et al., 2013).

A 6-month RCT performed in patients with AD showed no beneficial results in cognitive performances (Baum et al., 2008). More recently, a randomized clinical trial with Curcumin C3 Complex -consisting of 95% curcuminoids with 70–80% comprised by curcumin, 15–25% demethoxycurcumin, and 2.5–6.5% bisdemethoxycurcumin-was conducted in mild to moderate AD with no evident benefit on cognitive functions (Ringman et al., 2012).

CAROTENOIDS

Over 700 different members of the carotenoid family have been identified and about 40 are found in human blood and tissue. Major carotenoids in human organism are lycopene, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and the most prominent carotenoid, β-carotene. Depending on dietary habits, blood levels vary between 0.01 and 1 mol/L but they can be considerably increased upon supplementation with single compounds or mixtures (Stahl et al., 1992). For structural reasons and based on experimental data carotenoids have been assigned as antioxidants (Krinsky and Johnson, 2005). Carotenoids as natural fat-soluble pigments are found mostly in vegetables and fruits that are red, orange, and deep yellow in color. More recently, astaxanthin, a carotenoid mainly present in seafood, has been extensively studied in *in vitro* and *in vivo* models, showing antioxidant and anti-inflammatory properties as well as protective functions in microcirculation and in mitochondrial functions (Kidd, 2011) suggesting a potential efficacy in several neurodegenerative diseases (Barros et al., 2014). High plasma carotene concentrations associated with lower mortality from all causes were shown by the SENECA investigators (Buijsse et al., 2005), although conflicting data from intervention studies with β-carotene to prevent cancers and cardiovascular disorders have challenged the concept (Polidori and Stahl, 2009). Like other important carotenoids of the antioxidant network, lutein and zeaxanthin, the predominant carotenoids of the macula lutea, are suggested to act as photoprotectants preventing age-related degeneration of the macula lutea (Sabour-Pickett et al., 2012). Astaxanthin showed a protective effect for visual problems, such as blurred vision or reduced visual acuity, and also improved muscle strength and endurance in runners and in soccer players (Kidd, 2011).

Patients with moderate to severe AD showed lower plasma levels of two major carotenoids, lutein, and β-carotene, compared to patients with mild AD or controls (Wang et al., 2008b). Among AD patients a lower MMSE score (Mini Mental State Examination, a measure of cognitive performance) was associated with lower lutein and β-carotene levels in this study (Wang et al., 2008b). Lycopene displays not only sun protective effects (Stahl et al., 2006) but also beneficial effects against development of prostate cancer (Seren et al., 2008). Among six carotenoids tested, lycopene was the only carotenoid inversely associated with quality of cognitive performance as assessed by both MMSE, DemTect (Dementia Detection Test) and Clock Drawing Test in healthy subjects from 45 to 102 years of age (Polidori et al., 2009).

A randomized trial with beta carotene supplementation in men participating to the Physicians' Health Study showed, after a mean treatment duration of 18 years, better cognitive performances in the treated compared to the placebo group. The same effect was not observed in newly randomized subjects after one year treatment, suggesting that efficacy of beta carotene can be obtained only after a long term supplementation (Grodstein et al., 2007). In a multicenter trial in old age subjects suffering from MCI, a dietary supplement of astaxanthin, phosphatidylserine, and vitamin E improved memory skills after sixty days of treatment, suggesting a significant potential role of his kind of supplementation in counteracting age-related cognitive decline (Zanotta et al., 2014).

CROCIN – SAFFRON

Crocin is the main chemical compound identified in saffron, whose scientific name is *Crocus sativus*. It has been used over the years in folk medicine as an antispasmodic, gingival sedative, nerve sedative, carminative, and expectorant stimulant (Akhondzadeh and Abbasi, 2006; Akhondzadeh et al., 2010). It has been reported that saffron active constituents have anticonvulsant, antidepressant, and anti-inflammatory effects, and improve learning and memory (Schmidt et al., 2007). Crocin is the actual active component involved in both the improvement of learning and memory and preventing effect of long term potentiation blocked by ethanol (Akhondzadeh and Abbasi, 2006) and its potential in the treatment of neurodegenerative diseases like AD (Khalili and Hamzeh, 2010). In animal models, the effectiveness of crocin has been shown in antagonizing the cognitive deficits caused by neurotoxic agents like streptozocin (Naghizadeh et al., 2013).

Crocin improved cognition as evaluated by means of ADAS-Cog and CDR-SB in subjects with mild to moderate AD (Akhondzadeh et al., 2010). In a recent *in vivo* study, it has been demonstrated that crocin significantly modulate the levels of oxidative markers in the hippocampus, abolishing the deleterious effects of chronic stress on learning and memory (Ghadrdoost et al., 2011).

B-VITAMINS: FOLATE, COBALAMIN, PYRIDOXIN

Folate (vitamin B9), cobalamin (vitamin B12), and pyridoxin (vitamin B6) are the most studied B-vitamins in the field of cognitive decline and dementia (Brown et al., 2010). They are essential for maintaining the integrity of the nervous and hematopoietic systems (Malouf and Areosa Sastre, 2009).

Folate is absorbed from the diet and its decrease in blood is highly dependent on a poor diet, malabsorption, and alcoholism; cobalamin deficiency is also associated with malabsorption due to digestive disorders occurring in older adults, and can result in irreversible neurological disorders such as peripheral neuropathy (Rébeillé et al., 2007; Clarke, 2008). Vitamin B6 - comprising three chemically distinct compounds, pyridoxal, pyridoxamine, and pyridoxine - is involved in the regulation of mental function and mood. It is also an essential homocysteine re-methylation cofactor, and its deficiency is associated with an increase in blood homocysteine levels (Malouf and Grimley Evans, 2003). Poor vitamin B6 status is common among older people. Folate and cobalamine deficiencies cause accumulation of homocysteine. Many studies have found cross-sectional associations between low circulating folate levels or hyperhomocysteinemia and low MMSE scores (Morris and Jacques, 2009; Morris et al., 2012). Vitamin B12 is involved in the methylation of homocysteine to methionine for the synthesis of methyl acceptors such as membrane phospholipids, myelin and neurotransmitters. Homocysteine is potentially toxic to neurons, its levels have been associated with atrophic changes in the brain and are negatively correlated with neuropsychological tests scores; it is also considered a marker for low serum vitamin B12 and folate (Ellinson et al., 2004).

A prospective study over a 4, 5-year period found that homocysteine is a risk factor for dementia or cognitive impairment (Haan et al., 2007). In this study, plasma cobalamin levels were associated

with reduced risk (Haan et al., 2007). A recent clinical cohort trial found that plasma homocysteine levels are inversely related to cognitive performance, but no evidence of a significant protection of high plasma folate against dementia was found (Morris et al., 2012). However, B12- and B6-vitamin treatment has been demonstrated to stabilize performance on the CLOX test (Royall et al., 1998) as well as on executive and planning function (De Jager et al., 2012). Kado et al. (2005) and colleagues demonstrated that folate, but not cobalamin levels, are independently predictive of cognitive decline over a 7-year period in high functioning old people.

These heterogeneous and contradictory results are reported in the last Cochrane reviews, in which a significant effect of B-vitamin treatment in cognitive function could overall not be reported (Malouf and Grimley Evans, 2008; Malouf and Areosa Sastre, 2009).

Supplementation with B vitamins including vitamin B6 has been shown to reduce blood homocysteine levels. In addition, B6 vitamin concentration has been associated with better global cognition scores, especially with better attention, and executive function scores (Moorthy et al., 2012). Although vitamin B6 did not succeed in reducing atherosclerotic manifestations in hyperhomocysteinemic patients (Cacciapuoti, 2013), neuropsychiatric disorders - seizures, migraine, chronic pain, and depression - have been linked to vitamin B6 deficiency. However, there is no evidence that short-term treatment with vitamin B6 improves mood (depression, fatigue, and tension symptoms) or cognition (Malouf and Grimley Evans, 2003).

Recent RCTs on the effects of folate, vitamin B12, and vitamin B6 supplementation have been performed in subjects with mild to moderate AD. These failed in showing any effect of supplementation in slowing cognitive decline (Sun et al., 2007; Aisen et al., 2008; Malouf and Grimley Evans, 2008; Malouf and Areosa Sastre, 2009). A review on supplementation of vitamins B12, B6, and folic acid alone or in combination showed that results from nineteen RCTs did not appear to improve cognitive function in individuals with or without existing cognitive impairment (Ford and Almeida, 2012). So, it remains to be established if prolonged treatment with B-vitamins can reduce the risk of dementia in later life. However, in a recent study high-dose B-vitamin treatment (folic acid, vitamin B6, and vitamin B12) not only slowed shrinkage of the whole brain volume over 2 years but it reduced, by as much as sevenfold, the cerebral atrophy in those gray matter regions specifically vulnerable to the AD process, including the medial temporal lobe (Douaud et al., 2013).

DITERPENES: CARNOSIC ACID, AND ROSMARINIC ACID

Carnosic and rosmarinic acids are two of the most important antioxidant compounds in rosemary. They are parts of the bigger family of phenolic acids and diterpenes, that have been extensively

Table 2 | Nutraceuticals in clinical trials: reference, dosage, and study quality (*low; **moderate; ***high; ****very high).

	References	Dosage	Quality
Flavanols			
Catechin, epicatechin, epigallocatechin, EGCG	Krikorian et al. (2010a), Field et al. (2011), Scholey et al. (2010)	6–9 ml/kg of grape juice, 720 mg of cocoa flavanols, 300 mg EGCG	*, **, **
Flavonols			
Quercetin-kaempferol in Gingko biloba	Birks and Grimley Evans (2009; Cochrane)	80–720 mg /day of EGb761 (Gb extract)	****
Isoflavones			
Genistein, daidzein, glycetin in soy	Kreijkamp-Kaspers et al. (2004), Thorp et al. (2009), Henderson et al. (2012)	25.6 gr /day of soy protein, 116 mg/day isoflavone equivalent, 25 mg/day of soy protein	***, **, ***
Anthocyanidins			
Pelargonidin, cyanidine, malvidin in berries	Krikorian et al. (2010b), Devore et al. (2012)	6–9 ml/kg of blueberry juice, dietary intake of berries	*, ***
Resveratrol, curcumin	Kennedy et al. (2010), Ringman et al. (2012)	250 or 500 mg resveratrol, Curcumin C3 Complex 2–4 gr/day	*, **
Carotenoids			
Beta carotene, astaxanthin	Grodstein et al. (2007), Zanotta et al. (2014)	50 mg/alternate days, extract of astaxanthin	***, **
Crocin	Akhondzadeh et al. (2010)	30 mg/day saffron	**
Vitamin B6, vitamin B12, folic acid ±vit. B12, folic acid, vit.B12, vit. B6	Malouf and Grimley Evans (2003) (Cochrane), Malouf and Areosa Sastre (2009) (Cochrane), Malouf and Grimley Evans (2008) (Cochrane), Ford and Almeida (2012)	See reviewed studies	**, **, ***, ***

For the Cochrane reviews the quality refers to the mean quality of the reported studies.

studied (Yang et al., 2001; Kayashima and Matsubara, 2012). Both carnosic and rosmarinic acid showed a neuroprotective action both in *in vitro* models of neuronal death and in *in vivo* models of neurodegeneration. They scavenge reactive nitrogen species (Qiao et al., 2005; Kelsey et al., 2010) and protect neuroblastoma cells from hydrogen peroxide-induced oxidative stress (Lee et al., 2008; Kelsey et al., 2010).

Diterpenes also significantly alleviate memory impairment associated with Aβ neurotoxicity in AD and significantly delay the onset of the disease (Kelsey et al., 2010; Shimojo et al., 2010). Recently it has been found that carnosic acid has antiangiogenic effects (Kayashima and Matsubara, 2012). The neuroprotective effects of this substance, therefore, might be explained on the basis of the recently identified role of angiogenesis in the formation of β-amyloid plaques and the consequent neurotoxicity (Vagnucci and Li, 2003). The exact mechanism by which carnosinic acid inhibits angiogenesis is not clear; however, its antioxidant activity seems to play an important antiangiogenic role.

CONCLUSION AND FUTURE PERSPECTIVE

When placed in the context of a healthy lifestyle behavior, age-related changes in nutrition may play an important role in brain functioning as well as in major organ functioning of old people. Susceptibility of elderly population to specific nutrient deficits may exacerbate processes of cognitive decline. Indeed, there is large evidence regarding benefits of several nutrients in the common diet towards cognitive impairment and other diseases. There is great public and scientific interest about the potential of nutritional supplementation to prevent age-related diseases in general and cognitive decline in particular by counteracting deleterious neurodegenerative and pathological processes. We reviewed several components of common diets and several phytochemicals that have been shown to have benefits on these diseases and cognitive impairment. Unfortunately, there is a substantial lack of well conducted studies to be included in comparison analyses; scientific literature is still poor of RCTs (a summary is reported in **Table 2**) for effects of some of these molecules, and lots of studies are conducted on either *in vitro* models or animal models. A very few studies testing the effects of a combination of two substances or antioxidant mixtures also display little benefit against AD onset or progression as well as against the transition of MCI to AD. All critical components of studies on nutraceuticals in cognitive impairment, from sample size to cell types, to dosage to cognitive measures used, are not comparable. For instance, diagnostic criteria of MCI and AD as well as inclusion criteria and outcomes are not homogeneous. Start and end of the intervention with a particular nutraceutical in MCI or AD have been set for logistic reasons mostly so that intervention begin occurs too late during the course of the disease and study end delimits a far too short intervention period. Finally, specific compound-related issues might be responsible for the lack of clear success of intervention, including dosage-, kinetic-, bioavailability-, metabolic, and genetic-related issues that have been repeatedly discussed (http://lpi.oregonstate.edu/infocenter/cognition.html; Polidori and Schulz, 2014). Despite this, it seems difficult to uniform trial design and method in AD nutritional studies.

Theoretically, phytochemical-based treatments for geriatric cognitive decline and depression could be moved into a stronger clinical trial level on humans, especially due to their low toxicity and high bioavailability. Future studies addressing whether short-term or long-term dietary intake of nutraceuticals can reduce the severity and incidence of neurodegenerative and other age-related diseases appear critical and important for the future.

REFERENCES

Aherne, S. A., and O'Brien, N. M. (2002). Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75–81. doi: 10.1016/S0899-9007(01)00695-5

Aisen, P. S., Schneider, L. S., Sano, M., Diaz-Arrastia, R., van Dyck, C. H., Weiner, M. F., et al. (2008). High-dose B vitamin supplementation and cognitive decline in Alzheimer disease: a randomized controlled trial. *JAMA* 300, 1774–1783. doi: 10.1001/jama.300.15.1774

Akhondzadeh, S., and Abbasi, S. H. (2006). Herbal medicine in the treatment of Alzheimer's disease. *Am. J. Alzheimers Dis. Other Demen.* 21, 113–118. doi: 10.1177/153331750602100211

Akhondzadeh, S., Sabet, M. S., Harirchian, M. H., Togha, M., Cheraghmakani, H., Razeghi, S., et al. (2010). Saffron in the treatment of patients with mild to moderate Alzheimer's disease: a 16-week, randomized and placebo-controlled trial. *J. Clin. Pharm. Ther.* 35, 581–588. doi: 10.1111/j.1365-2710.2009.01133.x

Alvira, D., Yeste-Velasco, M., Folch, J., Verdaguer, E., Canudas, A. M., Pallàs, M., et al. (2007). Comparative analysis of the effects of resveratrol in two apoptotic models: inhibition of complex I and potassium deprivation in cerebellar neurons. *Neuroscience* 147, 746–756. doi: 10.1016/j.neuroscience.2007.04.029

Alzheimer's Association, (2009). Available at: http://www.alz.org/alzheimers_disease_facts_and_figures.asp

Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O., Lamuela-Raventos, R. M., and Joseph, J. A. (2005). Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutr. Neurosci.* 8, 111–120. doi: 10.1080/10284150500078117

Bansal, N., and Parle, M. (2010). Soybean supplementation helps reverse age- and scopolamine-induced memory deficits in mice. *J. Med. Food* 13, 1293–1300. doi: 10.1089/jmf.2010.1132

Barros, M. P., Poppe, S. C., and Bondan, E. F. (2014). Neuroprotective properties of the marine carotenoid astaxanthin and omega-3 fatty acids, and perspectives for the natural combination of both in krill oil. *Nutrients* 6, 1293–1317. doi: 10.3390/nu6031293

Baum, L., Lam, C. W., Cheung, S. K., Kwok, T., Lui, V., Tsoh, J., et al. (2008). Six-month randomized, placebo-controlled, double-blind, pilot clinical trial of curcumin inpatients with Alzheimer disease. *J. Clin. Psychopharmacol.* 28, 110–113. doi: 10.1097/jcp.0b013e318160862c

Bertelli, A. A., and Das, D. K. (2009). Grapes, wines, resveratrol, and heart health. *J. Cardiovasc. Pharmacol.* 54, 468–476. doi: 10.1097/FJC.0b013e3181bfaff3

Birks, J., and Grimley Evans, J. (2009). Ginkgo biloba for cognitive impairment and dementia. *Cochrane Database Syst. Rev.* CD003120. doi: 10.1002/14651858.CD003120.pub3

Brewer, G. J., Torricelli, J. R., Lindsey, A. L., Kunz, E. Z., Neuman, A., Fisher, D. R., et al. (2010). Age-related toxicity of amyloid-beta associated with increased pERK and pCREB in primary hippocampal neurons: reversal by blueberry extract. *J. Nutr. Biochem.* 21, 991–998. doi: 10.1016/j.jnutbio.2009.08.005

Brown, L. A., Riby, L. M., and Reay, J. L. (2010). Supplementing cognitive aging: a selective review of the effects of ginkgo biloba and a number of everyday nutritional substances. *Exp. Aging Res.* 36, 105–122. doi: 10.1080/03610730903417960

Buijsse, B., Feskens, E. J., Schlettwein-Gsell, D., Ferry, M., Kok, F. J., Kromhout, D., et al. (2005). Plasma carotene and alpha-tocopherol in relation to 10-y all-cause and cause-specific mortality in European elderly: the Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA). *Am. J. Clin. Nutr.* 82, 879–886.

Cacciapuoti, F. (2013). Lowering homocysteine levels with folic acid and B-vitamins do not reduce early atherosclerosis, but could interfere with cognitive decline and Alzheimer's disease. *J. Thromb. Thrombolysis* 36, 258–262. doi: 10.1007/s11239-012-0856-x

Calabrese, V., Cornelius, C., Mancuso, C., Barone, E., Calafato, S., Bates, T., et al. (2009). Vitagenes, dietary antioxidants and neuroprotection in neurodegenerative diseases. *Front. Biosci.* 14:376–397. doi: 10.2741/3250

Chan, P. C., Xia, Q., and Fu, P. P. (2007). Ginkgo biloba leave extract: biological, medicinal, and toxicological effects. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* 25, 211–244. doi: 10.1080/10590500701569414

Checkoway, H., Powers, K., Smith-Weller, T., Franklin, G. M., Longstreth, W. T. Jr., and Swanson, P. D. (2002). Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. *Am. J. Epidemiol.* 155, 732–738. doi: 10.1093/aje/155.8.732

Cheng, H. Y., Hsieh, M. T., Tsai, F. S., Wu, C. R., Chiu, C. S., Lee, M. M., et al. (2010). Neuroprotective effect of luteolin on amyloid beta protein (25-35)-induced toxicity in cultured rat cortical neurons. *Phytother. Res.* 24(Suppl 1), S102–S108. doi: 10.1002/ptr.2940

Chowdhury, A. R., Sharma, S., Mandal, S., Goswami, A., Mukhopadhyay, S., and Majumder, H. K. (2002). Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase I. *Biochem. J.* 366, 653–661. doi: 10.1042/BJ20020098

Clarke, R. (2008). B-vitamins and prevention of dementia. *Proc. Nutr. Soc.* 67, 75–81. doi: 10.1017/S0029665108006046

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., Abin-Carriquiry, J. A., Costa, G., et al. (2003). Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res.* 36, 1613–1620. doi: 10.1590/S0100-879X2003001200002

Dacks, P. A., Shineman, D. W., and Fillit, H. M. (2013). Current evidence for the clinical use of long-chain polyunsaturated n-3 fatty acids to prevent age-related cognitive decline and Alzheimer's disease. *J. Nutr. Health Aging* 17, 240–251. doi: 10.1007/s12603-012-0431-3

De Jager, C. A., Oulhaj, A., Jacoby, R., Refsum, H., and Smith, A. D. (2012). Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. *Int. J. Geriatr. Psychiatry* 27, 592–600. doi: 10.1002/gps.2758

De Kosky, S. T., Williamson, J. D., Fitzpatrick, A. L., Kronmal, R. A., Ives, D. G., Saxton, J. A., et al. (2008). Ginkgo biloba for prevention of dementia: a randomized controlled trial. *JAMA* 300, 2253–2262. doi: 10.1001/jama.2008.683

De la Torre, J. C. (2010). Vascular risk factor detection and control may prevent Alzheimer's disease. *Ageing Res. Rev.* 9, 218–25. doi: 10.1016/j.arr.2010.04.002

Denis, I., Potier, B., Vancassel, S., Heberden, C., and Lavialle, M. (2013). Omega-3 fatty acids and brain resistance to ageing and stress: body of evidence and possible mechanisms. *Ageing Res. Rev.* 12, 579–594. doi: 10.1016/j.arr.2013.01.007

Devore, E. E., Kang, J. H., Breteler, M. M., and Grodstein, F. (2012). Dietary intakes of berries and flavonoids in relation to cognitive decline. *Ann. Neurol.* 72, 135–143. doi: 10.1002/ana.23594

Djuric, Z., Chen, G., Doerge, D. R., Heilbrun, L. K., and Kucuk, O. (2001). Effect of soy isoflavone supplementation on markers of oxidative stress in men and women. *Cancer Lett.* 172, 1–6. doi: 10.1016/S0304-3835(01)00627-9

Dong, Z. H., Zhang, C. Y., and Pu, B. H. (2012). Effects of ginkgo biloba tablet in treating mild cognitive impairment. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 32, 1208–1211.

Douaud, G., Refsum, H., de Jager, C. A., Jacoby, R., Nichols, T. E., Smith, S. M., et al. (2013). Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9523–9528. doi: 10.1073/pnas.1301816110

Duncan, A. M., Phipps, W. R., and Kurzer, M. S. (2003). Phyto-oestrogens. *Best Pract. Res. Clin. Endocrinol. Metab.* 17, 253–271. doi: 10.1016/S1521-690X(02)00103-3

Dutta, K., Ghosh, D., and Basu, A. (2009). Curcumin protects neuronal cells from Japanese encephalitis virus-mediated cell death and also inhibits infective viral particle formation by dysregulation of ubiquitin-proteasome system. *J. Neuroimmune Pharmacol.* 4, 328–337. doi: 10.1007/s11481-009-9158-2

Eggler, A. L., Gay, K. A., and Mesecar, A. D. (2008). Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. *Mol. Nutr. Food Res.* 52(Suppl. 1), S84–S94. doi: 10.1002/mnfr.200700249

El Omri, A., Han, J., Kawada, K., Ben, A. M., and Isoda, H. (2012). Luteolin enhances cholinergic activities in PC12 cells through ERK1/2 and PI3K/Akt pathways. *Brain Res.* 1437, 16–25. doi: 10.1016/j.brainres.2011.12.019

Ellinson, M., Thomas, J., and Patterson, A. (2004). A critical evaluation of the relationship between serum vitamin B, folate and total homocysteine with cognitive impairment in the elderly. *J. Hum. Nutr. Diet.* 17, 371–383. doi: 10.1111/j.1365-277X.2004.00532.x

Everitt, A. V., Hilmer, S. N., Brand-Miller, J. C., Jamieson, H. A., Truswell, A. S., Sharma, A. P., et al. (2006). Dietary approaches that delay age-related diseases. *Clin. Interv. Aging* 1, 11–31. doi: 10.2147/ciia.2006.1.1.11

Féart, C., Samieri, C., Allès, B., and Barberger-Gateau, P. (2013). Potential benefits of adherence to the Mediterranean diet on cognitive health. *Proc. Nutr. Soc.* 72, 140–152. doi: 10.1017/S0029665112002959

Field, D. T., Williams, C. M., and Butler, L. T. (2011). Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions. *Physiol. Behav.* 103, 255–260. doi: 10.1016/j.physbeh.2011.02.013

File, S. E., Jarrett, N., Fluck, E., Duffy, R., Casey, K., and Wiseman, H. (2001). Eating soya improves human memory. *Psychopharmacology (Berl).* 157, 430–436. doi: 10.1007/s002130100845

Fisher, N. D., Sorond, F. A., and Hollenberg, N. K. (2006). Cocoa flavanols and brain perfusion. *J. Cardiovasc. Pharmacol.* 47(Suppl. 2), S210–S214. doi: 10.1097/00005344-200606001-00017

Ford, A. H., and Almeida, O. P. (2012). Effect of homocysteine lowering treatment on cognitive function: a systematic review and meta-analysis of randomized controlled trials. *J. Alzheimers Dis.* 29, 133–149. doi: 10.3233/JAD-2012-111739

Francis, S. T., Head, K., Morris, P. G., and Macdonald, I. A. (2006). The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. *J. Cardiovasc. Pharmacol.* 47(Suppl. 2), S215–S220. doi: 10.1097/00005344-200606001-00018

Frisardi, V., Panza, F., Solfrizzi, V., Seripa, D., and Pilotto, A. (2010). Plasma lipid disturbances and cognitive decline. *J. Am. Geriatr. Soc.* 58, 2429–2430. doi: 10.1111/j.1532-5415.2010.03164.x

Fuentealba, J., Dibarrart, A. J., Fuentes-Fuentes, M. C., Saez-Orellana, F., Quinones, K., Guzman, L., et al. (2011). Synaptic failure and adenosine triphosphate imbalance induced by amyloid-β aggregates are prevented by blueberry-enriched polyphenols extract. *J. Neurosci. Res.* 89, 1499–1508. doi: 10.1002/jnr.22679

Gabor, R., Nagle, R., Johnson, D. A., and Gibbs, R. B. (2003). Estrogen enhances potassium-stimulated acetylcholine release in the rat hippocampus. *Brain Res.* 962, 244–247. doi: 10.1016/S0006-8993(02)04053-2

Ganguli, M., Chandra, V., Kamboh, M. I., Johnston, J. M., Dodge, H. H., Thelma, B. K., et al. (2000). Apolipoprotein E polymorphism and Alzheimer disease: The Indo-US Cross-National Dementia Study. *Arch. Neurol.* 57, 824–830. doi: 10.1001/archneur.57.6.824

Ghadrdoost, B., Vafaei, A. A., Rashidy-Pour, A., Hajisoltani, R., Bandegi, A. R., Motamedi, F., et al. (2011). Protective effects of saffron extract and its active constituent crocin against oxidative stress and spatial learning and memory deficits induced by chronic stress in rats. *Eur. J. Pharmacol.* 667, 222–229. doi: 10.1016/j.ejphar.2011.05.012

Grant, W. B. (1999). Dietary links to Alzheimer's disease: 1999 update. *J. Alzheimers Dis.* 1, 197–201.

Grodstein, F., Kang, J. H., Glynn, R. J., Cook, N. R., and Gaziano, J. M. (2007). A randomized trial of beta carotene supplementation and cognitive function in men: the Physicians' Health Study II. *Arch. Intern. Med.* 167, 2184–2190. doi: 10.1001/archinte.167.20.2184

Haan, M. N., Miller, J. W., Aiello, A. E., Whitmer, R. A., Jagust, W. J., Mungas, D. M., et al. (2007). Homocysteine, B vitamins, and the incidence of dementia and cognitive impairment: results from the Sacramento Area Latino Study on Aging. *Am. J. Clin. Nutr.* 85, 511–517.

Hardy, G., Hardy, I., and Ball, P. A. (2003). Nutraceuticals – a pharmaceutical viewpoint: part II. *Curr. Opin. Clin. Nutr. Metab. Care* 6, 661–671. doi: 10.1097/00075197-200311000-00010

Harnly, J. M., Doherty, R. F., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Bhagwat, S., et al. (2006). Flavonoid content of U.S. fruits, vegetables, and nuts. *J. Agric. Food Chem.* 54, 9966–9977. doi: 10.1021/jf061478a

Harrison, F. E., Bowman, G. L., and Polidori, M. C. (2014). Ascorbic acid and the brain: rationale for the use against cognitive impairment. *Nutrients* 6, 1752–1781. doi: 10.3390/nu6041752

Henderson, V. W., St John, J. A., Hodis, H. N., Kono, N., McCleary, C. A., Franke, A. A., et al. (2012). Long-term soy isoflavone supplementation and cognition in women: a randomized, controlled trial. *Neurology* 78, 1841–1848. doi: 10.1212/WNL.0b013e318258f822

Heo, H. J., and Lee, C. Y. (2004). Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. *J. Agric. Food Chem.* 52, 7514–7517. doi: 10.1021/jf049243r

Heo, J. H., Hyon-Lee, and Lee, K. M. (2013). The possible role of antioxidant vitamin C in Alzheimer's disease treatment and prevention. *Am. J. Alzheimers Dis. Other Demen.* 28, 120–125. doi: 10.1177/1533317512473193

Hong, J. T., Yen, J. H., Wang, L., Lo, Y. H., Chen, Z. T., and Wu, M. J. (2009). Regulation of heme oxygenase-1 expression and MAPK pathways in response to kaempferol and rhamnocitrin in PC12 cells. *Toxicol. Appl. Pharmacol.* 237, 59–68. doi: 10.1016/j.taap.2009.02.014

Hu, G., Bidel, S., Jousilahti, P., Antikainen, R., and Tuomilehto, J. (2007). Coffee and tea consumption and the risk of Parkinson's disease. *Mov. Disord.* 22, 2242–2248. doi: 10.1002/mds.21706

Kado, D. M., Karlamangla, A. S., Huang, M. H., Troen, A., Rowe, J. W., Selhub, J., et al. (2005). Homocysteine versus the vitamins folate, B6, and B12 as predictors of cognitive function and decline in older high-functioning adults: MacArthur Studies of Successful Aging. *Am. J. Med.* 118, 161–167. doi: 10.1016/j.amjmed.2004.08.019

Kalra, E. K. (2003). Nutraceutical – definition and introduction. *AAPS Pharm. Sci.* 5, E25. doi: 10.1208/ps050325

Kalt, W., Foote, K., Fillmore, S. A., Lyon, M., Van Lunen, T. A., and McRae, K. B. (2008). Effect of blueberry feeding on plasma lipids in pigs. *Br. J. Nutr.* 100, 70–78. doi: 10.1017/S0007114507877658

Kanowski, S., and Hoerr, R. (2003). Ginkgo biloba extract EGb 761 in dementia: intent-to-treat analyses of a 24-week, multi-center, double-blind, placebo-controlled, randomized trial. *Pharmacopsychiatry* 36, 297–303. doi: 10.1055/s-2003-45117

Karlsson, J., Emgard, M., Brundin, P., and Burkitt, M. J. (2000). Trans-resveratrol protects embryonic mesencephalic cells from tert-butyl hydroperoxide: electron paramagnetic resonance spin trapping evidence for a radical scavenging mechanism. *J. Neurochem.* 75, 141–150. doi: 10.1046/j.1471-4159.2000.0750141.x

Kayashima, T., and Matsubara, K. (2012). Antiangiogenic effect of carnosic acid and carnosol, neuroprotective compounds in rosemary leaves. *Biosci. Biotechnol. Biochem.* 76, 115–119. doi: 10.1271/bbb.110584

Kelsey, N. A., Wilkins, H. M., and Linseman, D. A. (2010). Nutraceutical antioxidants as novel neuroprotective agents. *Molecules* 15, 7792–7814. doi: 10.3390/molecules15117792

Kennedy, D. O., Wightman, E. L., Reay, J. L., Lietz, G., Okello, E. J., Wilde, A., et al. (2010). Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. *Am. J. Clin. Nutr.* 91, 1590–1597. doi: 10.3945/ajcn.2009.28641

Khalili, M., and Hamzeh, F. (2010). Effects of active constituents of Crocus sativus L., crocin on streptozocin-induced model of sporadic Alzheimer's disease in male rats. *Iran Biomed. J.* 14, 59–65.

Kidd, P. (2011). Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Altern. Med. Rev.* 16, 355–364.

Kreijkamp-Kaspers, S., Kok, L., Grobbee, D. E., de Haan, E. H., Aleman, A., Lampe, J. W., et al. (2004). Effect of soy protein containing isoflavones on cognitive function, bone mineral density, and plasma lipids in postmenopausal women: a randomized controlled trial. *JAMA* 292, 65–74. doi: 10.1001/jama.292.1.65

Krikorian, R., Nash, T. A., Shidler, M. D., Shukitt-Hale, B., and Joseph, J. A. (2010a). Concord grape juice supplementation improves memory function in older adults with mild cognitive impairment. *Br. J. Nutr.* 103, 730–734. doi: 10.1017/S0007114509992364

Krikorian, R., Shidler, M. D., Nash, T. A., Kalt, W., Vinqvist-Tymchuk, M. R., Shukitt-Hale, B., et al. (2010b). Blueberry supplementation improves memory in older adults. *J. Agric. Food Chem.* 58, 3996–4000. doi: 10.1021/jf9029332

Krinsky, N. I., and Johnson, E. J. (2005) Carotenoid actions and their relation to health and disease. *Mol. Aspects Med.* 26, 459–516. doi: 10.1016/j.mam.2005.10.001

Kuriyama, S., Hozawa, A., Ohmori, K., Shimazu, T., Matsui, T., Ebihara, S., et al. (2006). Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project 1. *Am. J. Clin. Nutr.* 83, 355–361.

Larson, E. B., Yaffe, K., and Langa, K. M. (2013). New insights into the dementia epidemic. *New Engl. J Med.* 369, 2275–2277. doi: 10.1056/NEJMp1311405

Le Bars, P. L. (2003). Magnitude of effect and special approach to Ginkgo biloba extract EGb 761 in cognitive disorders. *Pharmacopsychiatry* 36(Suppl. 1), S44–S49. doi: 10.1055/s-2003-40458

Le Bars, P. L., Katz, M. M., Berman, N., Itil, T. M., Freedman, A. M., and Schatzberg, A. F. (1997). A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb Study Group. *JAMA.* 278, 1327–1332. doi: 10.1001/jama.1997.03550160047037

Lee, H., Kim, H. J., Kim, J. M., and Chang, N. (2004a). Effects of dietary folic acid supplementation on cerebrovascular endothelial dysfunction in rats with induced hyperhomocysteinemia. *Brain Res.* 996, 139–147. doi: 10.1016/j.brainres.2003.10.027

Lee, Y. B., Lee, H. J., Won, M. H., Hwang, I. K., Kang, T. C., Lee, J. Y., et al. (2004b). Soy isoflavones improve spatial delayed matching-to-place performance and reduce cholinergic neuron loss in elderly male rats. *J. Nutr.* 134, 1827–1831.

Lee, H. J., Cho, H. S., Park, E., Kim, S., Lee, S. Y., Kim, C. S., et al. (2008). Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. *Toxicology* 250, 109–15. doi: 10.1016/j.tox.2008.06.010

Lei, Y., Chen, J., Zhang, W., Fu, W., Wu, G., Wei, H., et al. (2012). In vivo investigation on the potential of galangin, kaempferol and myricetin for protection of D-galactose-induced cognitive impairment. *Food Chem.* 135, 2702–2707. doi: 10.1016/j.foodchem.2012.07.043

Lim, G. P., Chu, T., Yang, F., Beech, W., Frautschy, S. A., and Cole, G. M. (2001). The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J. Neurosci.* 21, 8370–8377.

Lin, P. Y., Chiu, C. C., Huang, S. Y., and Su, K. P. (2012). A meta-analytic review of polyunsaturated fatty acid compositions in dementia. *J. Clin. Psychiatry* 73, 1245–1254. doi: 10.4088/JCP.11r07546

Losi, G., Puia, G., Garzon, G., de Vuono, M. C., and Baraldi, M. (2004). Apigenin modulates GABAergic and glutamatergic transmission in cultured cortical neurons. *Eur. J. Pharmacol.* 502, 41–46. doi: 10.1016/j.ejphar.2004.08.043

Maclennan, K. M., Darlington, C. L., and Smith, P. E. (2002). The CNS effects of ginkgo biloba extracts and ginkgolide B. *Progr. Neurobiol.* 67, 235–257. doi: 10.1016/S0301-0082(02)00015-1

Malin, D. H., Lee, D. R., Goyarzu, P., Chang, Y. H., Ennis, L. J., Beckett, E., et al. (2011). Short-term blueberry-enriched diet prevents and reverses object recognition memory loss in aging rats. *Nutrition* 27, 338–342. doi: 10.1016/j.nut.2010.05.001

Malouf, R., and Areosa Sastre, A. (2009). Vitamin B12 for cognition. *Cochrane Database Syst. Rev.* CD004326.

Malouf, R., and Grimley Evans, J. (2003). The effect of vitamin B6 on cognition. *Cochrane Database Syst. Rev*. CD004393.

Malouf, R., and Grimley Evans, J. (2008). Folic acid with or without vitamin B12 for the prevention and treatment of healthy elderly and demented people. *Cochrane Database Syst. Rev.* CD004514. doi: 10.1002/14651858.CD004514.pub2

Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* 79, 727–747.

Mangialasche, F., Kivipelto, M., Mecocci, P., Rizzuto, D., Palmer, K., Winblad, B., et al. (2010). High plasma levels of vitamin E forms and reduced Alzheimer's disease risk in advanced age. *J. Alzheimers Dis.* 20, 1029–1037. doi: 10.3233/JAD-2010-091450

Mangialasche, F., Solomon, A., Kåreholt, I., Hooshmand, B., Cecchetti, R., Fratiglioni, L., et al. (2013). Serum levels of vitamin E forms and risk of cognitive impairment in a Finnish cohort of older adults. *Exp. Gerontol.* 48, 1428–1435. doi: 10.1016/j.exger.2013.09.006

Mangialasche, F., Xu, W., Kivipelto, M., Costanzi, E., Ercolani, S., Pigliautile, M., et al. (2012). Tocopherols and Tocotrienols plasma levels are associated with cognitive impairment. *Neurobiol. Aging* 33, 2282–2290. doi: 10.1016/j.neurobiolaging.2011.11.019

Mann, G. E., Rowlands, D. J., Li, F. Y., de Winter, P., and Siow, R. C. (2007). Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression. *Cardiovasc. Res.* 75, 261–274. doi: 10.1016/j.cardiores.2007.04.004

Mecocci, P., and Polidori, M. C. (2012). Antioxidant clinical trials in mild cognitive impairment and Alzheimer's disease. *Biochim. Biophys. Acta* 1822, 631–638. doi: 10.1016/j.bbadis.2011.10.006

Mecocci, P., Polidori, M. C., and Praticò, D. (2013). "Antioxidant clinical trials in mild cognitive impairment and alzheimer's disease," in *Studies on Alzheimer's Disease*, eds D. Praticò and P. Mecocci (New York, NY: Springer), 223–232.

Morris, M. S., and Jacques, P. F. (2009). "Folate and neurological function: Epidemiological perspective," in *Folate in Health and Disease*, ed. L. B. Bailey, Vol. 2 (Boca Raton, FL: CRC Press), 325–353.

Morris, M. S., Selhub, J., and Jacques, P. F. (2012). Vitamin B-12 and folate status in relation to decline in scores on the mini-mental state examination in the Framingham heart study. *J. Am. Geriatr. Soc.* 60, 1457–1464. doi: 10.1111/j.1532-5415.2012.04076.x

Moorthy, D., Peter, I., Scott, T. M., Parnell, L. D., Lai, C. Q., Crott, J. W., et al. (2012). Status of vitamins B-12 and B-6 but not of folate, homocysteine, and the methylenetetrahydrofolate reductase C677T polymorphism are associated with impaired cognition and depression in adults. *J. Nutr.* 142, 1554–1560. doi: 10.3945/jn.112.161828

Naghizadeh, B., Mansouri, M. T., Ghorbanzadeh, B., Farbood, Y., and Sarkaki, A. (2013). Protective effects of oral crocin against intracerebroventricular streptozotocin-induced spatial memory deficit and oxidative stress in rats. *Phytomedicine* 20, 537–542. doi: 10.1016/j.phymed.2012.12.019

Narita, K., Hisamoto, M., Okuda, T., and Takeda, S. (2011). Differential neuroprotective activity of two different grape seed extracts. *PLoS ONE* 6:e14575. doi: 10.1371/journal.pone.0014575

Ogle, W. O., Speisman, R. B., and Ormerod, B. K. (2013). Potential of treating age-related depression and cognitive decline with nutraceutical approaches: a mini-review. *Gerontology* 59, 23–31. doi: 10.1159/000342208

Orgogozo, J. M., Dartigues, J. F., Lafont, S., Letenneur, L., Commenges, D., Salamon, R., et al. (1997). Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Rev. Neurol.* 153, 185–192.

Panza, F., Solfrizzi, V., Colacicco, A. M., D'Introno, A., Capurso, C., Torres, F., et al. (2004). Mediterranean diet and cognitive decline. *Public Health Nutr.* 7, 959–963. doi: 10.1079/PHN2004561

Petrozzi, L., Ricci, G., Giglioli, N. J., Siciliano, G., and Mancuso, M. (2007). Mitochondria and neurodegeneration. *Biosci. Rep.* 27, 87–104. doi: 10.1007/s10540-007-9038-z

Pocernich, C. B., Lange, M. L., Sultana, R., and Butterfield, D. A. (2011). Nutritional approaches to modulate oxidative stress in Alzheimer's disease. *Curr. Alzheimer Res.* 8, 452–469. doi: 10.2174/156720511796391908

Polidori, M. C., Pientka, L., and Mecocci, P. (2012). A review of the major vascular risk factors related to Alzheimer's disease. *J. Alzheimers Dis.* 32, 521–530. doi: 10.3233/JAD-2012-120871

Polidori, M. C., Praticó, D., Mangialasche, F., Mariani, E., Aust, O., Anlasik, T., et al. (2009). High fruit and vegetable intake is positively correlated with antioxidant status and cognitive performance in healthy subjects. *J. Alzheimers Dis.* 17, 921–927. doi: 10.3233/JAD-2009-1114

Polidori, M. C., and Schulz, R. J. (2014). Nutritional contributions to dementia prevention: main issues on antioxidant micronutrients. *Genes Nutr.* 9, 382. doi: 10.1007/s12263-013-0382-2

Polidori, M. C., and Stahl, W. (2009). "Carotenoids and vitamin A," in *Chemoprevention of Cancer and DNA Damage by Dietary Factors*, eds I. Johnson, D. De Marini, C. Gerhäuser, and S. Knasmüller (Weinheim: Wiley International), 23.

Psaltopoulou, T., Sergentanis, T. N., Panagiotakos, D. B., Sergentanis, I. N., Kosti, R., and Scarmeas, N. (2013). Mediterranean diet, stroke, cognitive impairment, and depression: A meta-analysis. *Ann. Neurol.* 74, 580–591. doi: 10.1002/ana.23944

Qiao, S., Li, W., Tsubouchi, R., Haneda, M., Murakami, K., Takeuchi, F., et al. (2005). Rosmarinic acid inhibits the formation of reactive oxygen and nitrogen species in RAW264.7 macrophages. *Free Radic. Res.* 9, 995–1003. doi: 10.1080/10715760500231836

Ramirez, M. R., Izquierdo, I., do Carmo Bassols Raseira, M., Zuanazzi, J. A., Barros, D., and Henriques, A. T. (2005). Effect of lyophilised Vaccinium berries on memory, anxiety and locomotion in adult rats. *Pharmacol. Res.* 52, 457–462. doi: 10.1016/j.phrs.2005.07.003

Rébeillé, F., Ravanel, S., Marquet, A., Mendel, R. R., Webb, M. E., Smith, A. G., et al. (2007). Roles of vitamins B5, B8, B9, B12 and molybdenum cofactor at cellular and organismal levels. *Nat. Prod. Rep.* 24, 949–962. doi: 10.1039/b703104c

Rendeiro, C., Guerreiro, J. D., Williams, C. M., and Spencer, J. P. (2012). Flavonoids as modulators of memory and learning: molecular interactions resulting in behavioural effects. *Proc. Nutr. Soc.* 71, 246–262. doi: 10.1017/S0029665112000146

Ringman, J. M., Frautschy, S. A., Teng, E., Begum, A. N., Bardens, J., Beigi, M., et al. (2012). Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. *Alzheimer Res. Therapy* 4, 43. doi: 10.1186/alzrt146

Royall, D. R., Cordes, J. A., and Polk, M. (1998). CLOX: an executive clock drawing task. *J. Neurol. Neurosurg. Psychiatry* 64, 588–594. doi: 10.1136/jnnp.64.5.588

Sabour-Pickett, S., Nolan, J. M., Loughman, J., and Beatty, S. (2012). A review of the evidence germane to the putative protective role of the macular carotenoids for age-related macular degeneration. *Mol. Nutr. Food Res.* 56, 270–286. doi: 10.1002/mnfr.201100219

Sasaki, N., Toda, T., Kaneko, T., Baba, N., and Matsuo, M. (2003). Protective effects of flavonoids on the cytotoxicity of linoleic acid hydroperoxide toward rat pheochromocytoma PC12 cells. *Chem. Biol. Interact.* 145, 101–116. doi: 10.1016/S0009-2797(02)00248-X

Schmidt, M., Betti, G., and Hensel, A. (2007). Saffron in phytotherapy: pharmacology and clinical uses. *Wien Med. Wochenschr.* 157, 315–319. doi: 10.1007/s10354-007-0428-4

Scholey, A. B., French, S. J., Morris, P. J., Kennedy, D. O., Milne, A. L., and Haskell, C. F. (2010). Consumption of cocoa flavanols results in acute improvements in mood and cognitive performance during sustained mental effort. *J. Psychopharmacol.* 24, 1505–1514. doi: 10.1177/0269881109106923

Schültke, E., Kendall, E., Kamencic, H., Ghong, Z., Griebel, R. W., and Juurlink, B. H. (2003). Quercetin promotes functional recovery following acute spinal cord injury. *J. Neurotrauma* 20, 583–591. doi: 10.1089/089771503767168500

Seren, S., Lieberman, R., Bayraktar, U. D., Heath, E., Sahin, K., Andic, F., et al. (2008). Lycopene in cancer prevention and treatment. *Am. J. Ther.* 15, 66–81. doi: 10.1097/MJT.0b013e31804c7120

Sharma, V., Mishra, M., Ghosh, S., Tewari, R., Basu, A., Seth, P., et al. (2007). Modulation ofinterleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. *Brain Res. Bull.* 73, 55–63. doi: 10.1016/j.brainresbull.2007.01.016

Shimojo, Y., Kosaka, K., Noda, Y., Shimizu, T., and Shirasawa, T. (2010). Effect of rosmarinic acid in motor dysfunction and life span in a mouse model of familial amyotrophic lateral sclerosis. *J. Neurosci. Res.* 88, 896–904. doi: 10.1002/jnr.22242

Shukitt-Hale, B., Lau, F. C., Carey, A. N., Galli, R. L., Spangler, E. L., Ingram, D. K., et al. (2008). Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutr. Neurosci.* 11, 172–82. doi: 10.1179/147683008X301487

Singh, B., Parsaik, A. K., Mielke, M. M., Erwinc, P. J., Knopman, D. S., Petersen, R. C., et al. (2013). Association of Mediterranean diet with mild cognitive impairment and Alzheimer's disease: a systematic review and meta-analysis. *J. Alzheimers Dis.* 39, 271–282. doi: 10.3233/JAD-130830

Snitz, B. E., O'Meara, E. S., Carlson, M. C., Arnold, A. M., Ives, D. G., Rapp, S. R., et al. (2009). Ginkgo biloba for preventing cognitive decline in older adults: a randomized trial. *JAMA* 302, 2663–2670. doi: 10.1001/jama.2009.1913

Sofi, F., Cesari, F., Abbate, R., Gensini, G. F., and Casini, A. (2008). Adherence to Mediterranean diet and health status: meta-analysis. *Br. Med. J.* 337, a1344aq. doi: 10.1136/bmj.a1344

Spencer, J. P. (2009). The impact of flavonoids on memory: physiological and molecular considerations. *Chem. Soc. Rev.* 38, 1152–1161. doi: 10.1039/b800422f

Spencer, J. P. (2010). Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain. *Proc. Nutr. Soc.* 69, 244–260. doi: 10.1017/S0029665110000054

Spencer, J. P., Abd El Mohsen, M. M., Minihane, A. M., and Mathers, J. C. (2008). Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br. J. Nutr.* 99, 12–22. doi: 10.1017/S0007114507798938

Stahl, W., Heinrich, U., Aust, O., Tronnier, H., and Sies, H. (2006). Lycopene-rich products and dietary photoprotection. *Photochem. Photobiol. Sci.* 5, 238–242. doi: 10.1039/b505312a

Stahl, W., Schwarz, W., Sundquist, A. R., and Sies, H. (1992). cis-trans Isomers of lycopene and b-carotene in human serum and tissues. *Arch. Biochem. Biophys.* 294, 173–177. doi: 10.1016/0003-9861(92)90153-N

Sun, G. Y., Xu, J., Jensen, M. D., and Simonyi, A. (2004). Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J. Lipid Res.* 45, 205–213. doi: 10.1194/jlr.R300016-JLR200

Sun, Y., L, C. J., Chien, K. L., Chen, S. T., and Chen, R. C. (2007). Efficacy of multivitamin supplementation containing vitamins B6 and B12 and folic acid as adjunctive treatment with a cholinesterase inhibitor in Alzheimer's disease: a 26-week, randomized, double-blind, placebo-controlled study in Taiwanese patients. *Clin. Ther.* 29, 2204–2214. doi: 10.1016/j.clinthera.2007.10.012

Sydenham, E., Dangour, A. D., and Lim, W. S. (2012). Omega 3 fatty acid for the prevention of cognitive decline and dementia. *Cochrane Database. Syst. Rev.* 6, D005379. doi: 10.1002/14651858.CD005379.pub3

Thorp, A. A., Sinn, N., Buckley, J. D., Coates, A. M., and Howe, P. R. (2009). Soya isoflavone supplementation enhances spatial working memory in men. *Br. J. Nutr.* 102, 1348–1354. doi: 10.1017/S0007114509990201

Truelsen, T., Thudium, D., Grønbaek, M., and Copenhagen City Heart Study. (2002). Amount and type of alcohol and risk of dementia: the Copenhagen City Heart Study. *Neurology* 59, 1313–1319. doi: 10.1212/01.WNL.0000031421.0369.E7

Tsuda, T. (2008). Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. *J. Agric. Food. Chem.* 56, 642–646. doi: 10.1021/jf073113b

Vagnucci, A. H. Jr., and Li, W. W. (2003). Alzheimer's disease and angiogenesis. *Lancet* 361, 605–608. doi: 10.1016/S0140-6736(03)12521-4

Vellas, B., Coley, N., Ousset, P. J., Berrut, G., Dartigues, J. F., Dubois, B., et al. (2012). Long-term use of standardised Ginkgo biloba extract for the prevention of Alzheimer's disease (GuidAge): a randomised placebo-controlled trial. *Lancet Neurol.* 11, 851–859. doi: 10.1016/S1474-4422(12)70206-5

Wang, J., Ho, L., Zhao, W., Ono, K., Rosensweig, C., Chen, L., et al. (2008a). Grape-derived polyphenolics prevent Abeta oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's disease. *J. Neurosci.* 28, 6388–6392. doi: 10.1523/JNEUROSCI.0364-08.2008

Wang, W., Shinto, L., Connor, W. E., and Quinn, J. F. (2008b). Nutritional biomarkers in Alzheimer's disease: the association between carotenoids, n-3 fatty acids, and dementia severity. *J. Alzheimers Dis.* 13, 31–38.

Wang, M. H., Chang, W. J., Soung, H. S., and Chang, K. C. (2012). (-)-Epigallocatechin-3-gallate decreases the impairment in learning and memory in spontaneous hypertension rats. *Behav. Pharmacol.* 23, 771–780. doi: 10.1097/FBP.0b013e32835a3bc8

Williams, C. M., El Mohsen, M. A., Vauzour, D., Rendeiro, C., Butler, L. T., Ellis, J. A., et al. (2008). Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. *Free Radic. Biol. Med.* 45, 295–305. doi: 10.1016/j.freeradbiomed.2008.04.008

Williams, R. J., and Spencer, J. P. (2012). Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free Radic. Biol. Med.* 52, 35–45. doi: 10.1016/j.freeradbiomed.2011.09.010

Xu, Y., Barish, P. A., Pan, J., Ogle, W. O., and O'Donnell, J. M. (2012). Animal models of depression and neuroplasticity: assessing drug action in relation to behavior and neurogenesis. *Methods Mol. Biol.* 829, 103–124. doi: 10.1007/978-1-61779-458-2_6

Xu, Y., Wang, Z., You, W., Zhang, X., Li, S., Barish, P. A., et al. (2010). Antidepressant-like effect of trans-resveratrol: Involvement of serotonin and noradrenaline system. *Eur. Neuropsychopharmacol.* 20, 405–413. doi: 10.1016/j.euroneuro.2010.02.013

Yang, C. S., Landau, J. M., Huang, M. T., and Newmark, H. L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* 21, 381–406. doi: 10.1146/annurev.nutr.21.1.381

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., and Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* 51, 675–683. doi: 10.1002/mnfr.200700002

Zanotta, D., Puricelli, S, and Bonoldi, G. (2014). Cognitive effects of a dietary supplement made from extract of Bacopa monnieri, astaxanthin, phosphatidylserine, and vitamin E in subjects with mild cognitive impairment: a noncomparative, exploratory clinical study. *Neuropsychiatr. Dis. Treat.* 10, 225–230. doi: 10.2147/NDT.S51092

Zhao, L., and Brinton, R. D. (2007). WHI and WHIMS follow-up and human studies of soy isoflavones on cognition. *Expert. Rev. Neurother.* 7, 1549–1564. doi: 10.1586/14737175.7.11.1549

Zhao, L., Wang, J. L., Wang, Y. R., and Fa, X. Z. (2013). Apigenin attenuates copper-mediated β-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model. *Brain Res.* 1492, 33–45. doi: 10.1016/j.brainres.2012.11.019

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From industrial research to academic discoveries, toward a new concept of partnership: the Biomathics model

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A commentary on

The battle of Alzheimer's Disease - the beginning of the future Unleashing the potential of academic discoveries
by Lundkvist, J., Halldin, M. M., Sandin, J., Nordvall, G., Forsell, P., Svensson, S., et al. (2014). Front. Pharmacol. 5:102. doi: 10.3389/fphar.2014.00102

Recently, Winblad and colleagues (Lundkvist et al., 2014) reported an interesting point of view highlighting the current failures of the pharmaceutical industry to find an effective drug solution against Alzheimer's disease, and the difficulties rising from greater Research and Development costs in the context of growing regulatory and administrative straitjacket. This paradigm shift may result to a critical situation of decline of Alzheimer's research and therapy. Alzheimer's disease is a worldwide big concern because of its expanding prevalence and incidence, and its numerous adverse consequences including loss of autonomy, poor quality of life, high morbid-mortality and institutionalization (Prince et al., 2013). No curative treatment has been discovered so far, and the only treatment options are symptomatic (Anand et al., 2014). Thus, in order to reduce Alzheimer's impact and costs, the development of curative drugs proves necessary.

One solution emphasized by Winblad and colleagues to avoid this crisis is the back to basics way, meaning turning to non-profit academic research to break the deadlock (Lundkvist et al., 2014). The value of academic research is based on the small size of research teams, avoiding too much inertia and making it possible to work through close interaction rather than contracting; on the opportunity to test candidate molecules; and also on their ability to work on rare diseases and orphans drugs in the CNS area that may provide indirect solutions to the problem of Alzheimer's disease (Lundkvist et al., 2014). Eventually, most importantly, these biomedical academic research teams have the opportunity to develop more easily translational research and to conduct action-research, a way of thinking together and combining research and clinical practice (Beauchet et al., 2012).

The academic approach is indeed still promising. Nevertheless, it is worth pointing out that research as conceived hitherto could not answer a number of important issues, leaving the door open for the pharmaceutical industry. It is therefore highly suitable to make academic research evolve. The main problem was the excessive fragmentation, dispersion and confinement of skills and knowledge fiercely guarded by every academic research team. Research should overcome these difficulties and open up to others. Future of research will undoubtedly be based on the pooling of resources, research, databases, and teams. This is exactly the purpose of Biomathics consortium.

Biomathics is an emerging scientific research consortium applied to human systems modeling intended to help physicians and scientists worldwide to work together and think faster and wider. Firmly focused on the fields of human longevity, autonomy and prediction of health issues and their adverse consequences, Biomathics Consortium aims at generating initiatives, linking data from research and data from quantified-self, i.e., the self-measure of health and function using digital technologies to promote health in the general population (den Braber, 2013). Biomathics involves researchers worldwide, operates international collaborations, interconnects research databases on health and well-being, and initiates and/or joins international research programs.

Biomathics connects academic research teams working in the same research fields, and offer them to share their respective basis in order to compound a larger and more comprehensive data basis. Of course, this organization process requires to evaluate similar or close outcomes, and to adopt a shared language. Nevertheless, methods based on effect size or z-score can combine slightly different variables. This allows extremely fast answers to research questions with only little additional financial resources and using very large population-based samples. Moreover, it is also likely that some endpoints identified in a specific study may be of concern to another team, or at least may respond to queries of this second team. In such case, the requesting team launches an initiative within Biomathics and contacts all teams likely to help. Willing researchers are included in the initiative, participate in the research, revise the collaborative publication and

are included in the list of co-authors based on their involvement in the work and the number of participants made available.

The main strengths of Biomathics Consortium are to make a formal link between worldwide leading research teams on aging and longevity, specifically those who have not worked together thus far. It allows building an effective worldwide operational network, applying to grants as a research network, training students and fellows, and offering immediate reactivity and high efficiency. It offers data extracted from various international clinical researches. It makes also possible to screen for potential participants for future research programs. Last, but not least, it promotes the emergence of new ideas and the validation of pilot projects through international multicentric studies. For this reason, Biomathics Consortium is a typical and distinctive model of "bottom-up" operation in the context of "top-down" reorganization of most research programs due to budgetary constraints and restrictions. While research studies are increasingly built to meet government programs and grants ("top-down" model), Biomathics Consortium is raised by researchers for themselves and is intended to explore research questions that do not necessarily receive the attention of governing bodies. In convergence with top-down organization, rapid production of big results would eventually influence the decision-making and change health and research policies, hence the "bottom-up" word acceptation.

In conclusion, we are at a crucial time for research. Times are changing, research too. Tomorrow's opportunities imply global convergence, efficient networks, and merging academic legitimacy and flexibility with private funds and stringency to keep producing innovative science not restricted by commercial objectives. Biomathics Consortium is intended to unify forces in order to improve the efficiency of research and quickly find effective treatment options for patients. Since this is still, and always must be, researchers' first priority.

AUTHOR CONTRIBUTIONS

All authors meet all of the following criteria: (1) contributing to the conception and design, or analyzing and interpreting data; (2) drafting the article or revising it critically for important intellectual content; and (3) approving the final version to be published.

REFERENCES

Anand, R., Gill, K. D., and Mahdi, A. A. (2014). Therapeutics of Alzheimer's disease: past, present and future. *Neuropharmacology* 76, 27–50. doi: 10.1016/j.neuropharm.2013.07.004

Beauchet, O., Fantino, B., and Annweiler, C. (2012). The 'action-research' philosophy: from bedside to bench, to bedside again. *Int. J. Clin. Pract.* 66, 517. doi: 10.1111/j.1742-1241.2012.02909.x

den Braber, M. (2013). Quantified Self: insight in yourself through self-monitoring. *Ned. Tijdschr. Geneeskd.* 157:A7028.

Lundkvist, J., Halldin, M. M., Sandin, J., Nordvall, G., Forsell, P., Svensson, S., et al. (2014). The battle of Alzheimer's Disease - the beginning of the future Unleashing the potential of academic discoveries. *Front. Pharmacol.* 5:102. doi: 10.3389/fphar.2014.00102

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., and Ferri, C. P. (2013). The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimers Dement.* 9, 63–75. doi: 10.1016/j.jalz.2012.11.007

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