# Flavonoids as Therapeutic Compounds Targeting Key Proteins Involved in Alzheimer's Disease

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ABSTRACT: Alzheimer's disease is characterized by pathological aggregation of protein tau and amyloid-β peptides, both of which are considered to be toxic to neurons. Naturally occurring dietary flavonoids have received considerable attention as alternative candidates for Alzheimer's therapy taking into account their antiamyloidogenic, antioxidative, and anti-inflammatory properties. Experimental evidence supports the hypothesis that certain flavonoids may protect against Alzheimer's disease in part by interfering with the generation and assembly of amyloid-β peptides into neurotoxic oligomeric aggregates and also by reducing tau aggregation. Several mechanisms have been proposed for the ability of flavonoids to prevent the onset or to slow the progression of the disease. Some mechanisms include their interaction with important signaling pathways in the brain like the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways that regulate prosurvival transcription factors and gene expression. Other processes include the disruption of amyloid-β aggregation and alterations in amyloid precursor protein processing through the inhibition of β-secretase and/or activation of α-secretase, and inhibiting cyclin-dependent kinase-5 and glycogen synthase kinase-3β activation, preventing abnormal tau phosphorylation. The interaction of flavonoids with different signaling pathways put forward their therapeutic potential to prevent the onset and progression of Alzheimer's disease and to promote cognitive performance. Nevertheless, further studies are needed to give additional insight into the specific mechanisms by which flavonoids exert their potential neuroprotective actions in the brain of Alzheimer's disease patients.

KEYWORDS: Flavonoids, Alzheimer's disease, amyloid precursor protein, amyloid beta, BACE-1, tau, signaling
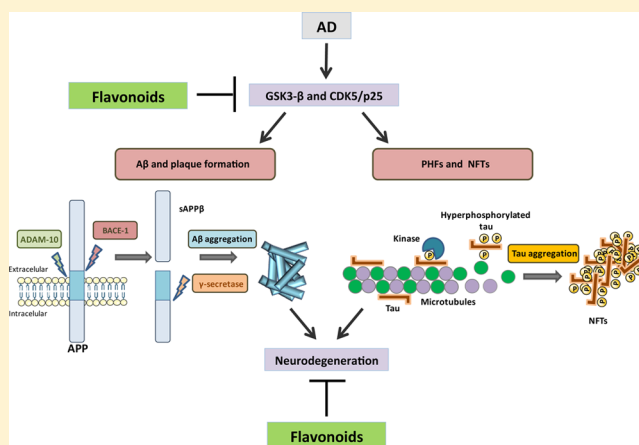
Alzheimer's disease (AD) is a neurodegenerative disorder and the most common form of dementia worldwide. The major histopathological hallmarks of AD include proteinous aggregates in the form of neurofibrillary tangles (NFTs), consisting of hyperphosphorylated tau[1,2] and extracellular senile plaques, which are deposits of heterogeneously sized small peptides of amyloid-β (Aβ) that are formed via sequential proteolytic cleavages of the amyloid precursor protein (APP)[3] (Figure 1). Dominant mutations in APP, presenilin-1 (PS1) or PS2 are responsible for the early onset or familial form of AD. These mutations have been shown to profoundly alter APP metabolism, favoring the production of aggregation-prone Aβ species, these findings formed the basis for the "amyloid cascade hypothesis" of AD pathogenesis. This broadly accepted hypothesis states that the generation of neurotoxic Aβ peptides by β-secretase and γ-secretase are at the basis of AD pathophysiology. Other hallmarks of this disease, like neurotransmitter changes[4,5] and neuronal and synapse loss in the neocortex and the hippocampus[6,7] develop as a consequence of this event.

AMYLOID PRECURSOR PROTEIN

APP belongs to a protein family that includes APP-like protein 1 and 2 in mammals.[8,9] All are single-pass transmembrane proteins with large extracellular domains, and all are similarly processed. Though the family shares several other conserved domains such as the E1 and E2 domains in the extracellular sequence, the Aβ domain is unique to the APP protein. Alternative splicing of the APP transcript generates eight isoforms, of which three are most common: the 695 amino acid form, which is expressed predominantly in the central nervous system, and the 751 and 770 amino acid forms, which are more ubiquitously expressed.[10] APP is synthesized in the endoplasmic reticulum and then transported through the Golgi
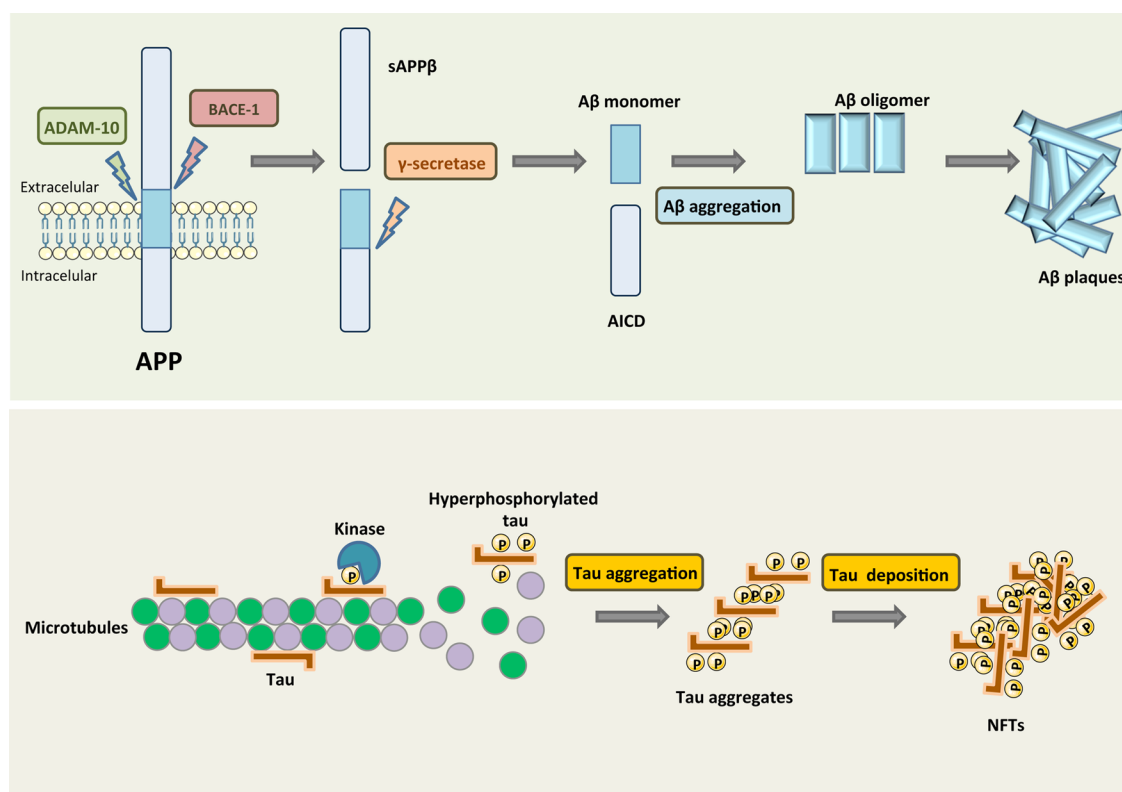
Figure 1. β-Amyloid plaques and neurofibrillary tangles are hallmark deposits of Alzheimer's disease. The major protein component of the plaques is Aβ that results from APP by proteolytic cleavage. β-Secretase (BACE-1) generates the amino terminus of Aβ, and γ-secretase defines its length. Aβ forms toxic oligomeric aggregates that then deposit as plaques. In AD, tau is hyperphosphorylated and dissociates from MTs, causing them to depolymerize. Tau is then deposited in aggregates such as NFTs.

apparatus to the trans-Golgi-network (TGN) where the highest concentration of APP is found in neurons at steady state.[11,12] From the TGN, APP can be transported in TGN-derived secretory vesicles to the cell surface where it can be proteolytically cleaved directly by α-secretase and then γ-secretase, producing a soluble molecule (sAPPα),[13] a process that does not generate Aβ. Alternatively, it can be reinternalized in clathrin-coated pits into another endosomal compartment containing the proteases β-secretase 1 (BACE-1) and γ-secretase,[14,15] resulting in the production of Aβ, which is then released into the extracellular space following vesicle recycling or otherwise degraded in lysosomes.

BACE-1 SECRETASE AND APP CLEAVAGE

The first step in Aβ generation is APP cleavage by the β-secretase; BACE-1 is the major β-secretase.[16,17] BACE-1 is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus.[16] Overexpression or downregulation of BACE-1 induces or inhibits cleavage of APP, confirming that BACE-1 is the β-secretase involved in APP metabolism and its activity is the rate-limiting factor in Aβ generation.[16,18]

Several studies have investigated the potential of BACE-1 as a therapeutic target. BACE-1 knockout mice do not produce detectable levels of Aβ and have no severe phenotypic abnormalities.[19,20] Suppression of this secretase by RNA interference reduced APP processing and Aβ production in primary cortical neurons derived from both wild-type and Swedish APP mutant transgenic mice.[21] Moreover, disruption of the BACE-1 gene rescued memory deficits and cholinergic

dysfunction in Swedish APP mice.[22] Studies have also found that BACE-1 protein and activity levels are elevated in brain regions affected by AD[23,24] and that oral administration of a potent and selective BACE-1 inhibitor decreased β-cleavage and Aβ production in APP transgenic mice in vivo.[25] Nevertheless, some studies also show that BACE-1 as a drug target may not be as safe as first assumed. For example, BACE-1 KO mice were reported to present hypomyelination of peripheral nerves and aberrant axonal segregation,[26] suggesting that inhibition of β-secretase may have unwanted serious collateral effects.

TAU PROTEIN AND ALZHEIMER'S DISEASE

Tau is one of the microtubule associated proteins that is thought to have a role in the stabilization of neuronal microtubules, providing the tracks for intracellular transport. In AD, tau protein is not able to keep the cytoskeleton well organized in the axonal process, since this protein loses its capacity to bind to microtubules due to conformational changes and misfoldings,[27,28] leading to its aberrant aggregation as fibrillary structures inside neurons.[29] Other tau modifications related to this dementia include phosphorylation, proteolysis, and ubiquitination, where abnormal phosphorylation is considered the most critical modification. Tau aggregation into paired helical filaments (PHFs) results from its hyper-phosphorylated state, and culminates in the formation of NFTs which constitute one of the earliest AD markers (Figure 1). Moreover, abnormal hyperphosphorylated tau detached from microtubules also leads to increased intraneuronal soluble tau
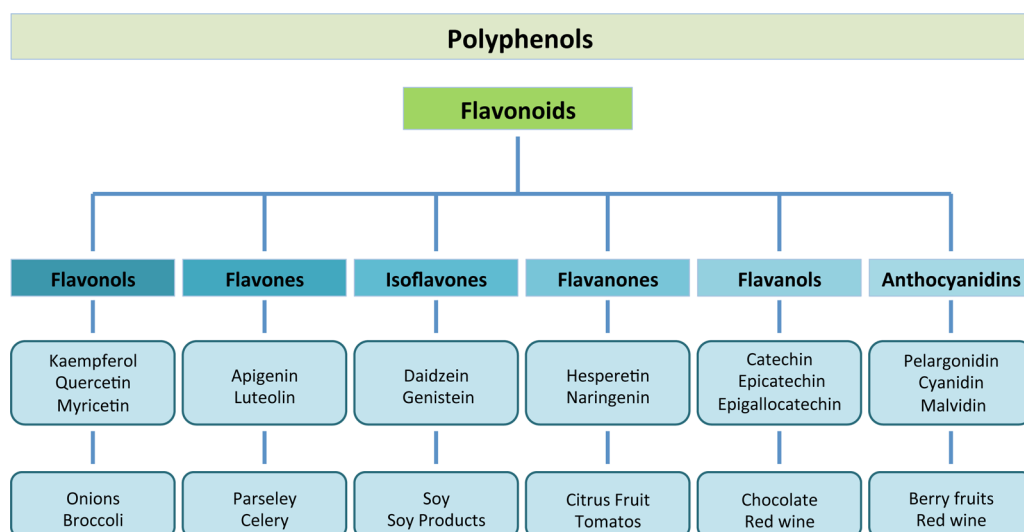
Figure 2. Major classes of natural flavonoids and their dietary sources.

concentration, due to sequestration of normal tau from microtubules, further facilitating tau aggregation into PHFs.[30]

Changes in tau protein, affecting stabilization of microtubules, are likely to impair axonal transport,[31,32] leading to changes in synaptic proteins and mitochondria axonal transport and ultimately culminating in "dying back" axons.

■ KINASES AND PHOSPHATASES: INVOLVEMENT IN ALZHEIMER'S DISEASE

In AD pathogenesis, the proteins that bind and interact with APP are different according to the phosphorylation state of APP.[33,34] Several studies have been performed to clarify the role of APP phosphorylation at specific residues. Within the APP molecule, Thr[668] is considered the major phosphorylation site, although other amino acids in the APP cytoplasmic domain are also phosphorylated.[35−37] CDK5 (cyclin-dependent kinase-5) and GSK-3β (glycogen synthase kinase-3β) are thought to phosphorylate APP at Thr[668] in neurons[38−40] and when cells are subjected to a stress stimulus, c-Jun N-terminal kinase (JNK) may also phosphorylate APP at Thr[668].[41,42] Hyperphosphorylation of APP in AD patients' brain may also be explained by the Aβ inhibition of phosphoprotein phosphatase 1 (PPP1)[35] or PPP2, thus contributing to the phosphorylation of APP at Thr668.[43]

As already referred, another hallmark in AD is the hyperphosphorylation of tau protein (Figure 1), and phosphorylation of tau regulates its binding activity to microtubules stimulating their assembly. Basal phosphorylation levels are required for optimal tau function, whereas, as mentioned previously, in an hyperphosphorylated state tau loses its biological activity. Around 85 tau phosphorylation sites, of which 28 are exclusively phosphorylated in AD brains, have been described.[44] In AD, abnormal tau phosphorylation may be the result of upregulation of tau kinase(s) or downregulation of tau phosphatase(s), although they may not be mutually exclusive.[45] The kinases that are assumed to play the most significant role in brain tau phosphorylation are GSK-3β, CDK5, cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase II (CaMK-II).[46] Among these kinases, GSK-3β may play a major role in regulating tau phosphorylation in both physiological and pathological conditions. GSK-3β can phosphorylate tau at

several residues and a complementary and PPP1, PPP2A, PPP2B, and PPP2C are all possible candidates that can dephosphorylate tau protein.[47] In general tau phosphoprotein is at least three- to 4-fold more hyperphosphorylated in the brain of AD patients when compared to that of aged nondemented individuals.[48]

■ FLAVONOIDS

An increasing body of evidence demonstrates the neuroprotective potential of flavonoids either by preventing the onset or by slowing the progression of age-related neurodegenerative diseases. Dietary supplementation studies using flavonoid-rich plant or food extracts have shown their ability to influence cognition and learning in humans and also in animal models of diseases.[49−55] Presently, there is no direct association between flavonoid consumption and improvement in neurological health. Nevertheless, the potential beneficial effect of flavonoids in the brain seems to be related to their ability to interact with intracellular neuronal and glial signaling pathways, thus influencing the peripheral and cerebral vascular system, protecting vulnerable neurons, enhancing existing neuronal function, or stimulating neuronal regeneration.[54]

Flavonoids are naturally occurring polyphenolic compounds widely spread in plants. They are present in foods and beverages of plant origin such as a variety of fruits, vegetables, cocoa, cereals, tea and wine.[56] The six main subclasses of flavonoids include the (1) flavonols (e.g., kaempferol, quercetin), which are present in onions, leeks, and broccoli; (2) flavones (e.g., apigenin, luteolin), present in parsley and celery; (3) isoflavones (e.g., daidzein, genistein), which are mainly found in soy and soy products; (4) flavanones (e.g., hesperetin, naringenin), mainly found in citrus fruit and tomatoes; (5) flavanols (e.g., catechin, epicatechin, epigallocatechin, epigallocatechin gallate (EGCG)), which are abundant in green tea, red wine, and chocolate; and finally (6) anthocyanidins (e.g., pelargonidin, cyanidin, malvidin), whose sources include berry fruits and also red wine[57] (Figure 2).

It was thought that the ability of flavonoids to promote memory, learning, and cognitive function was mediated by their antioxidant capacity.[58] Nevertheless, due to their limited absorption and their low bioavailability in the brain, increasing evidence demonstrates that they are able to interact with the
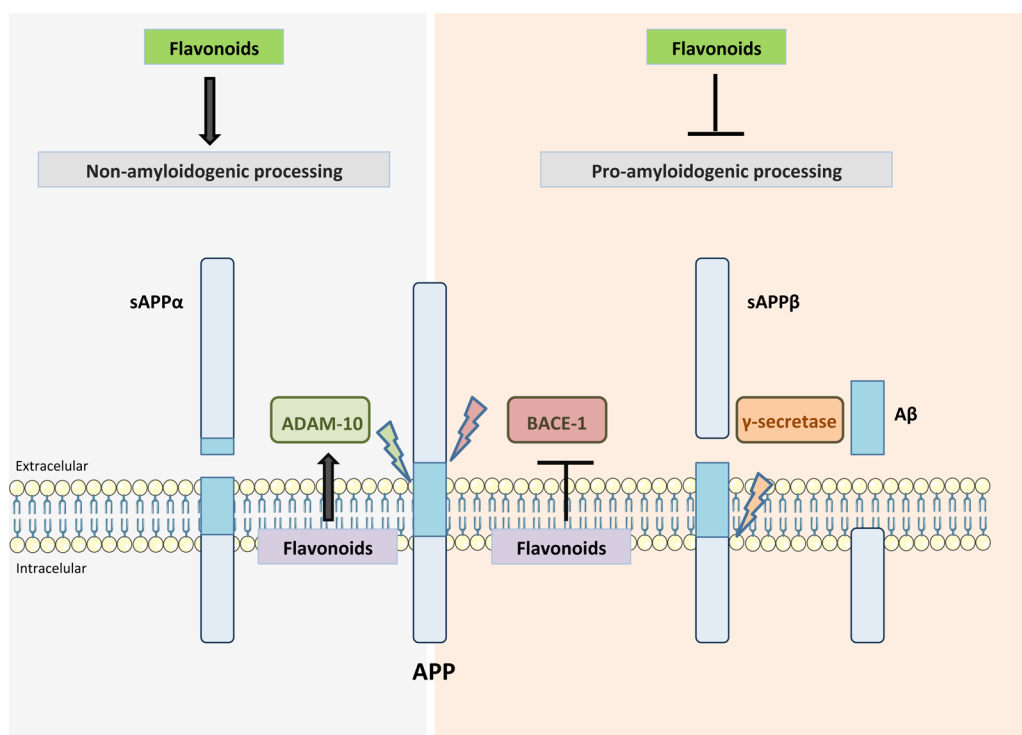
Figure 3. APP processing and flavonoid activity. Flavonoids can reduce Aβ production either by enhancing α-secretase (ADAM10) activity or by inhibiting β-secretase (BACE-1). Additionally, flavonoids may lead to the production of off-target Aβ oligomers, thereby disrupting fibrillization.

cellular and molecular components of the brain responsible for memory, having the potential to protect vulnerable neurons, enhance existing neuronal function, stimulate neuronal regeneration, and induce neurogenesis.[58,59]

■ EFFECT OF FLAVONOIDS ON APP PROCESSING

Several flavonoids have been shown to inhibit the development of AD and to reverse cognitive deficits in rodent models, indicating their potential therapeutic utility. Since altered APP processing leading to increased Aβ production is a key pathogenic feature of AD, several studies have been directed toward the antiamyloidogenic properties of flavonoids. In this regard, it was recently demonstrated that anthocyanin-enriched bilberry and black currant extracts have the ability to modulate APP processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of AD.[60] In the transgenic PSAPP mouse model of cerebral amyloidosis, oral administration of tannic acid for 6 months prevented transgene-associated behavioral impairment and defective spatial reference memory. Several other studies further support the efficacy of flavonoids on memory and learning, as for example, nobiletin, a citrus flavonoid, which proved to ameliorate Aβ-induced memory impairment and decrease the Aβ burden and plaques in the hippocampus of a transgenic mouse model of AD.[61] Moreover, grape derived polyphenols (GSPE) administered orally for 5 months to Tg2576 mice, attenuates cognitive deterioration coincidently with reduced levels of high-molecular-weight soluble Aβ oligomers in the brain.[62]

Another citrus flavonoid, luteolin, was shown to reduce Aβ peptide generation in both human "Swedish" mutant APP transgene-bearing neuron-like cells and primary neurons, also decreasing the amyloidogenic gamma-secretase APP processing.[63] Additionally, consumption of polyphenol-rich grape seed

extract or curcumin for 9 months prevented amyloid-beta deposition in the brain of an AD mouse model.[64]

At the APP processing level, it was demonstrated that long-term treatment (16 months) with Ginkgo biloba extract (EGb761) significantly lowered APP protein levels in a transgenic mouse model of AD, suggesting that its potential neuroprotective properties may be, at least partly, related to its APP lowering effects.[65] Also, brain parenchymal and cerebral vascular β-amyloid deposits were diminished in tannic acid treated PSAPP mice, suggesting that it acts as a natural β-secretase inhibitor.[66] Natural flavonoids were shown to potently inhibit BACE-1 activity and reduce the level of secreted Aβ in primary cortical neurons,[67] whereas epigallocatechin-3-gallate and curcumin suppress amyloid beta-induced BACE-1 upregulation in neuronal cultures.[68]

Several studies have focused on studying the beneficial properties of regular intake of green tea. Green tea polyphenol ($-$)-epigallocatechin-3-gallate (EGCG) has proved to have a beneficial role in reducing brain Aβ levels, by modulating amyloid precursor protein (APP) processing.[69,70] ADAM10 activation is necessary for EGCG promotion of nonamyloidogenic (α-secretase cleavage) APP processing.[71] Furthermore, EGCG-mediated enhancement of nonamyloidogenic processing of APP was found to be mediated by the maturation of ADAM10, via an estrogen receptor-α/phosphoinositide 3-kinase/Ak-transforming dependent mechanism. Since estrogen depletion following menopause has been correlated with an increased risk of developing AD, selective estrogen receptor modulation could be a therapeutic target and the use of EGCG could be considered as an alternative to estrogen therapy in the prophylaxis and treatment of this disease.[72]

EGCG may also have a neuroprotective action by possessing the ability to inhibit the formation of β-sheet-rich amyloid fibrils. It was demonstrated that it inhibits the fibrillogenesis of

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dx.doi.org/10.1021/cn400213r | ACS Chem. Neurosci. 2014, 5, 83−92

Aβ by directly binding to the natively unfolded polypeptides and preventing their conversion into on-pathway aggregation, intermediates which are toxic to neurons.[73] Moreover, EGCG has the ability to convert large amyloid-beta fibrils into smaller, amorphous protein aggregates that are nontoxic and therefore suggesting that EGCG is a potent remodeling agent of mature amyloid fibrils.[74] Besides EGCG, other flavonoids have also shown antiamyloidogenic properties especially myricetin, that exerts an antiamyloidogenic effect in vitro by preferentially and reversibly binding to the amyloid fibril structure of Aβ, rather than to Aβ monomers.[75,76]

Overall, these studies suggest that certain flavonoids are able to disrupt fibrillization by leading to the production of off-target Aβ oligomers, function as BACE-1 inhibitors or act by enhancing ADAM10 activity, consequently reducing Aβ production (Figure 3). However, more studies are needed to discover which flavonoid structures have the greatest beneficial potential and their underlying mechanisms of action.

■ TAU AND FLAVONOIDS

Potential beneficial effects of flavonoids in AD may play a role in downstream targets such as tau phosphorylation. Concerning this, some studies have elucidated aspects on the effect of flavonoids in the tau protein, which may impact AD. Myrecetin and epicatechin-5-gallate have been shown to inhibit heparin-induced tau formation[77] and EGCG administration in Alzheimer transgenic mice leads to modulated tau profiles, with suppression of sarkosyl-soluble phosphorylated tau isoforms.[70]

Other studies using GSPE have also shown its capability to inhibit tau neuropathology in a mouse model of AD, inhibiting tau peptide aggregations, as well as dissociating preformed tau peptide aggregates and disrupting PHFs.[78−81] Interaction of flavonoids with signaling pathways important for tau phosphorylation will be further discussed in the next section.

■ FLAVONOIDS AND THEIR INTERACTION WITH SIGNALING PATHWAYS

There is extensive evidence indicating that certain flavonoids[82] and some of their metabolites[83−85] are capable of exerting beneficial effects on neurological processes through their interaction with neuronal signaling pathways.

Potential flavonoid-binding sites on neurons include the adenosine,[86] GABA$_A$,[87−89] δ-opioid,[90,91] nicotinic,[92,93] TrkB,[94] estrogen,[72] and testosterone receptors,[95] as well as a specific brain plasma membrane binding site for polyphenols.[96] Flavonoids and their metabolites have been shown to exert neuronal effects through their interactions with a number of protein kinase and lipid kinase signaling cascades, such as the PI3K/Akt, tyrosine kinase, protein kinase C, and MAPK signaling pathways and the nuclear factor-κB pathway.[58,59,83,97−103] Inhibitory or stimulatory actions at these pathways are likely to greatly affect neuronal function, by changing the phosphorylation state of target molecules and/or by modulating gene expression. Consequently, this can lead to changes in plasticity, synaptic protein synthesis, and morphological changes involved in neurodegenerative processes and memory. Mitogen-activated kinases (MAPKs) belong to the superfamily of serine/threonine kinases and regulate several cellular mechanisms by transducing extracellular signals into intracellular responses.[104,105] It has been suggested that flavonoids and their metabolites may interact selectively with

the MAPK signaling pathways.[102,106] The action of flavonoids on the ERK pathway[83,99,107−110] appears to be mediated by interactions with MAPK kinases MEK1 and MEK2 and potentially membrane receptors.[102,111,112] In fact, flavonoids have close structural homology to specific pharmacological modulators of ERK signaling such as PD98059 (2′-amino-3′-methoxyflavone). Moreover, activation of ERK can result in downstream activation of cAMP response element binding protein (CREB), which may lead to changes in synaptic plasticity and memory[113,114] and to upregulation of neuroprotective pathways. It was demonstrated that memory performance in rats supplemented with blueberry, which contains high amounts of flavanols and anthocyanins, correlates with the activation of CREB and with increases in both pro- and mature levels of BDNF in the hippocampus,[115] both of which are linked to the control of synaptic plasticity and long-term memory. Moreover, administration of green tea catechins for 6 months prevented spatial learning and memory impairments in senescence-accelerated mouse prone-8 mice by decreasing Aβ (1−42) oligomers, increasing the activity of the protein kinase A/cAMP-response element binding protein (PKA/CREB) pathway, and by upregulating synaptic plasticity-related proteins in the hippocampus.[116]

Additionally flavonoids modulate PI3-kinase, via direct interactions with its ATP binding site.[98,117] One of the most selective PI3-kinase inhibitors available, LY294002, was modeled on the structure of quercetin.[100,118] LY294002 and quercetin fit into the binding pocket of the enzyme although with different orientations.[119] Substitution of hydroxyl groups on the flavonoid B ring and the degree of unsaturation of the C2−C3 bond in the C ring are important determinants of this particular bioactivity. Regarding this, it appears that different flavonoids are likely to express different cellular outcomes depending on their degree of interaction with either receptors or downstream kinases, meaning that the interactions with signaling pathways may be structure-dependent. One example of this is the flavonol quercetin and some of its in vivo metabolites which were shown to inhibit prosurvival Akt/PKB signaling pathways by inhibiting PI3-kinase activity,[83] whereas flavanones, such as hesperetin, have been shown to activate Akt/PKB signaling to confer prosurvival properties in cortical neurons.[108] Furthermore, it has been shown that EGCG stimulates extracellular signal-regulated kinase (ERK)- and PI3K-dependent increase in CREB phosphorylation and upregulates GluR2 levels in cortical neurons and can therefore act as a modulator in neurotransmission, plasticity, and synaptogenesis.[107]

Supplementing the diet of aged animals with blueberry for 12 weeks, has been shown to induce the phosphorylation of hippocampal Akt, the activation downstream of mTOR, and the increased expression of activity-regulated cytoskeletal-associated protein (Arc/Arg3.1).[115] Since Arc is known to be important in LTP and has been proposed to be under regulatory control of both BDNF,[120] such changes may underlie events related to spatial memory through the facilitation of alterations in synaptic strength, and the induction of morphological changes.[121] These possibly include alterations in neuronal spine density and morphology, which are considered vital for learning and memory.[122] Studies indicating that changes in neuronal morphology can occur in response to flavonoid supplementation support this hypothesis,[50,123] and certain flavonoids can influence neuronal dendrite outgrowth in vitro.[124]

Tau hyperphosphorylation and accumulation in neurofibrillary tangles is strongly correlated with cognitive deficits, and among the kinases that phosphorylate tau, GSK-3β is strongly implicated in AD pathogenesis. Flavonoids have proved to have beneficial effects by inhibiting the activity of certain kinases that contribute to this pathology. It was demonstrated that indirubins inhibit GSK-3β and CDK5/p25; both of these protein kinases are involved in abnormal tau phosphorylation in AD [125] (Figure 4). Moreover, the flavonoid
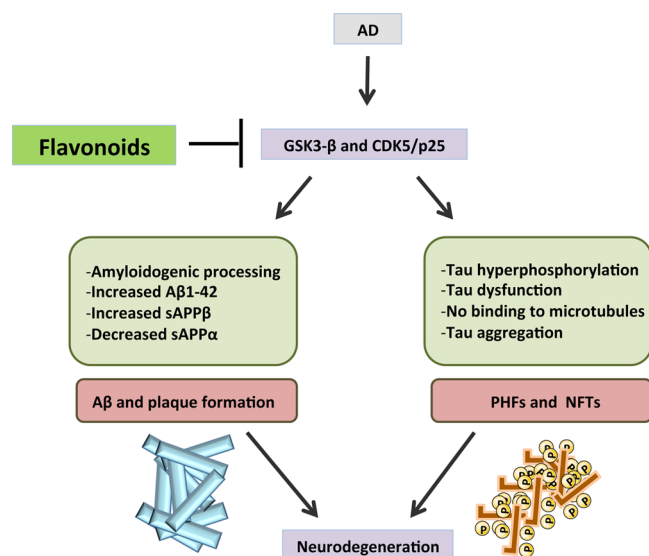

Figure 4. Flavonoids beneficial effects are exerted by inhibiting the activity GSK-3β and CDK5/p25, therefore preventing the activation of signaling cascades that contribute to neurodegeneration in AD.

morin is capable of inhibiting GSK-3β activity and blocking GSK-3β-induced tau phosphorylation in vitro. Morin also attenuated Aβ-induced tau phosphorylation and protected human neuroblastoma cells against Aβ cytotoxicity. Treatment of 3xTg-AD mice with morin resulted in a decrease in tau hyperphosphorylation in hippocampal neurons.[126] In the Tg2576 mouse model of AD, luteolin is able to decrease soluble Aβ levels, reduce GSK-3 activity, and disrupt PS1-APP association,[63] and recently it was demonstrated that cyanidin 3-O-glucoside (Cy3G) can rescue the cognitive impairments that are induced by Aβ via the modulation of GSK-3β/tau in rats, suggesting a potential therapeutic role of Cy3G in AD.[127] It seems reasonable to deduce that imbalances in the phosphorylation system are therefore one of the causes for hyperphosphorylation of cytoskeletal proteins in AD.

Although there has been intense interest in the ability of flavonoids to modulate kinases, there is no indication that they may affect signaling pathways via a modulation of phosphatase activity. Considering that phosphatases reverse the activity of kinases and since phosphatases are integral to many signaling pathways, it is conceivable that changes in ERK activation and related transcription factors may result from flavonoid-induced modulation of phosphatase activity.[82] Nevertheless, future studies are needed to evaluate the potential of flavonoids to inhibit, or activate phosphatases and their mechanisms of action.

CONCLUSIONS

Flavonoids are widely available in natural foods, and as a result, treatments for AD with such natural compounds through diet or dietary supplements are considered an attractive alternative. Flavonoids have demonstrated to have beneficial properties against the general mechanisms of AD in a variety of cell culture and animal models. Nevertheless, more studies addressing the specific mechanisms by which flavonoids exert their potential neuroprotective actions are required, before novel flavonoid-based dietary applications are applied in practice to reduce AD risk. Advances in the understanding of the mechanisms underlying flavonoid—protein interactions in AD, may represent a promising goal for developing novel neuroprotective strategies for neurodegenerative diseases.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS

AD, Alzheimer's disease; NFTs, neurofibrillary tangles; Aβ, amyloid-β peptide; APP, amyloid precursor protein; PS1, presenilin-1; TGN, trans-Golgi-network; BACE-1, beta-secretase 1; PHFs, Paired helical filaments; CDK5, cyclin-dependent kinase-5; GSK-3β, glycogen synthase kinase-3β; PPP1, phosphoprotein phosphatase 1; EGCG, (-)-epigallocatechin-3-gallate; ADAM10, A disintegrin and metalloproteinase domain-containing protein 10

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dx.doi.org/10.1021/cn400213r | ACS Chem. Neurosci. 2014, 5, 83−92