Review

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Multifunctional Activities of Green Tea Catechins in Neuroprotection

Modulation of Cell Survival Genes, Iron-Dependent Oxidative Stress and PKC Signaling Pathway

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Abstract
Many lines of evidence suggest that oxidative stress resulting in reactive oxygen species (ROS) generation and inflammation play a pivotal role in the age-associated cognitive decline and neuronal loss in neurodegenerative diseases including Alzheimer's (AD), Parkinson's (PD) and Huntington's diseases. One cardinal chemical pathology observed in these disorders is the accumulation of iron at sites where the neurons die. The buildup of an iron gradient in conjunction with ROS (superoxide, hydroxyl radical and nitric oxide) are thought to constitute a major trigger in neuronal toxicity and demise in all these diseases. Thus, promising future treatment of neurodegenerative diseases and aging depends on availability of effective brain permeable, iron-chelatable/radical scavenger neuroprotective drugs that would prevent the progression of neurodegeneration. Tea flavonoids (catechins) have been reported to possess potent iron-chelating, radical-scavenging and anti-inflammatory activities and to protect neuronal death in a wide array of cellular and animal models of neurological diseases. Recent studies have indicated that in addition to the known antioxidant activity of catechins, other mechanisms such as modulation of signal transduction pathways, cell survival/death genes and mitochondrial function, contribute significantly to the induction of cell viability. This review will focus on the multifunctional properties of green tea and its major component (–)-epigallocatechin-3-gallate (EGCG) and their ability to induce neuroprotection and neurorescue in vitro and in vivo. In particular, their transitional metal (iron and copper) chelating property and inhibition of oxidative stress.

Introduction

Polyphenols are natural substances present in beverages obtained from plants, fruits and vegetables such as olive oil, red wine and tea. Flavonoids are the largest group of polyphenols, which include the subclasses of flavones, isoflavones, flavanols, flavans and flavonols [1]. Several prototypes of these groups have been shown to promote a number of physiological benefits, especially in cognitive function and memory impairment. Fresh tea

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(Camellia sinensis) leaves contain a high amount of catechins, a group of flavonoids or flavanols, known to constitute 30–45% of the solid green tea extract [2, 3]. The favorable properties ascribed to tea consumption are believed to rely on its bioactive components, catechins and their derivatives, demonstrated to act directly as radical scavengers and exert indirect antioxidant effects through activation of transcription factors and antioxidant enzymes [for reviews, see 4, 5]. The most abundant polyphenolic compound is EGCG, thought to contribute to the beneficial effects attributed to green tea, such as its anticancer, cardiovascular function improvement and antioxidant anti-inflammatory properties. Indeed, a number of epidemiological studies have shown that phenolic compounds reduce the risk of coronary heart disease, possibly via their anti-inflammatory effects, including inhibition of adhesion molecule and cytokine expression and augmentation of endothelial nitric oxide release [6]. Relative antioxidant activities among tea catechins have been found to be EGCG = (–)-epicatechin-3-gallate (ECG) > (–)-epigallocatechin (EGC) > (–)-epicatechin (EC) [7]. EGCG accounts for more than 10% of the extract dry weight (30–130 mg per cup of tea) followed by EGC > EC ≥ ECG [2]. In addition to their radical scavenging action, green tea catechins possess well-established metal-chelating properties. Structurally important features defining their chelating potential are the 3′,4′-dihydroxyl group in the B ring [8] as well as the gallate group [9, 10], which may neutralize ferric iron to form redox-inactive iron, thereby protecting cells against oxidative damage [11]. However, a wealth of data is accumulating and indicating that the antioxidant/metal chelating attributes of the catechin polyphenols are unlikely to be the sole explanation for their neuroprotective and neurorescue capacity. Thus, catechin polyphenols were found to invoke a wide spectrum of different mechanisms of action responsible for cell survival [for our recent reviews, see 12, 13].

There is evidence that polyphenol metabolites and their parent compounds have access to the brain. Studies with radioactively labeled EGCG in mouse or chemiluminescence-based detection of EGCG in rats demonstrated its incorporation into brain, as well as in various organs including kidney, heart, liver, spleen and pancreas [14, 15]. Furthermore, it has been shown that the methylated and glucuronidated derivatives of epicatechin are both detected in rat brain following oral administration [16].

Significant body of evidence support the hypothesis that brain iron dysregulation and oxidative stress (OS), resulting in ROS generation from H_2O_2 and inflammatory processes, trigger a cascade of events leading to apoptotic/necrotic cell death in neurodegenerative disorders, such as Parkinson's (PD), Alzheimer's (AD) and Huntington's diseases and amyotrophic lateral sclerosis (ALS) [17]. There is also evidence for increased expression of apoptotic proteins (for review see [18]), as well as mitochondria (complex I) and ubiquitin-proteasome system (UPS) dysfunction, which may lead to breakdown of energy metabolism and consecutive intraneuronal calcium overload [19–22]. Thus, neurodegeneration appears to be multifactorial, where a complex set of reactions lead to the demise of neurons. This assumption receives support from the familial (genetic) forms of neurodegenerative diseases identified in the last years, where mutations in genes, such as α-synuclein, parkin and ubiquitin C-terminal hydrolase-L1 (UCHL-1) described in rare forms of hereditary PD [23], may lead to impairment in the activity of the UPS. More recently, recessive mutations in DJ-1 [24] and PINK1 (PTEN-induced kinase 1) [25] were proposed to play a role in cellular response to OS, supporting a pathogenic role of ROS in the etiology of neurodegenerative diseases. Therefore, it is not surprising that antioxidants were the first drugs to be studied in an attempt to retard the progress of PD. Recently, coenzyme Q_{10}, an intrinsic component of the mitochondrial respiratory chain acting as a bioenergizer and an antioxidant, was studied as a putative neuroprotective agent in PD. This double-blind, placebo-controlled pilot study demonstrated that high doses of coenzyme Q_{10} (1,200 mg/d) were associated with a reduced rate of deterioration in motor function from baseline over the 16-month course of this trial [26].

Neuropathological and neurochemical studies on substantia nigra (SN) from PD brains and its animal models [23, 27, 28] and our recent gene expression profiling of human SN pars compacta (pc) from PD patients [29] demonstrate the existence of a 'domino' cascade of neurotoxic events, which can be initiated at any point in the cascade. These series of events may act independently or cooperatively during the course of the disease, leading eventually to the demise of dopaminergic neurons. This has led to the current notion that drugs directed against a single target will be ineffective and rather a single drug or cocktail of drugs with pluripharmacological properties may be more suitable to be employed. According to this belief, green tea catechins well fulfill the requirements for a putative neuroprotective drug having diverse pharmacological activities. Thus, it is not surprising that they

have attracted increasing interest as therapeutic cytoprotective agents for the treatment of neurodegenerative and other diseases.

In this article the state of the art of the diverse molecular mechanisms and cell signaling pathways participating in the neuroprotective action of green tea catechin polyphenols is reviewed. Particular attention has been paid to their iron-chelating properties with respect to the potential promise for iron chelation therapy, as a novel treatment for neurodegenerative diseases.

Neuroprotective Effects of Catechins: Insights from in vivo and in vitro Studies

There is a growing recognition that polyphenolic catechins exert a protective role in neurodegeneration. An experimental study conducted in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD has shown that both green tea extract and EGCG effectively prevent mice striatal dopamine (DA) depletion and SN dopaminergic neuron loss [30]. The protection exerted by green tea polyphenols in vivo may involve direct scavenging of ROS and regulation of antioxidant protective enzymes. EGCG was found to elevate the activity of two major oxygen-radical species metabolizing enzymes, superoxide dismutase (SOD) and catalase in mice striatum [30]. This is supported by a previous finding where 1 month's administration of a catechin-containing antioxidant preparation increased SOD activity in the mitochondria fraction of striatum and midbrain and decreased thiobarbiturate reactive substance formation in the cortex and cerebellum of aged rats [31]. The structural catechol resemblance of EGCG may explain a recently reported inhibitory effect of green tea polyphenols on the DA presynaptic transporters. This inhibition lead to 1-methyl-4-phenylpyridinium (MPP^+) uptake blockade (because of competition for the vesicular transporter) thereby protecting DA-containing neurons against $MPP^{(+)}$-induced injury [32]. In addition, EGCG greatly inhibited catechol-O-methyltransferase (COMT) activity in rat liver cytosol at a low IC_{50} concentration (0.2 μM) [33]. This action may be of particular significance for PD patients, given that DA and related catecholamines are physiological substrates of COMT, thus its inhibition will result in increased DA in the synapse.

Green tea polyphenols have been also shown beneficial in animal models of cerebral ischemia: intraperitoneal injection of EGCG reduced hippocampal neuronal damage and brain edema caused by global [34] or unilateral [35] cerebral ischemia in gerbils. Insights into the possible mechanism of neuroprotection by EGCG in the infarct area of ischemic rats, revealed that it acts by reducing iNOS expression, infiltration and peroxynitrite formation [36], by increasing endothelial and neuronal NOS and preservation of mitochondrial complex activity and integrity [37]. In this context, the decrease in the activity of the transcription factor signal transducer and activator of transcription-1alpha (STAT-1alpha) by EGCG in ischemic rat cardiac myocytes may well account for the reduced mRNA levels of iNOS, a target of STAT-1 [38]. Other investigators have recently shown that EGCG reduced brain inflammation and neuronal damage in experimental autoimmune encephalomyelitis (EAE), when given at initiation or after the onset of EAE [39].

An extensive number of studies regarding neuroprotection by green tea flavonoids in cellular and animal models of neurodegenerative diseases are starting to accumulate. Hence, the flavonoid epicatechin was shown to attenuate the toxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons [40] or fibroblasts [41] and to confer protection to primary culture of mesencephalic neurons challenged with 6-hydroxydopamine (6-OHDA) [42]. Recently, catechin was shown to reduce injury produced by hydrogen peroxide, 4-hydroxynonenal, rotenone and 6-OHDA in primary rat mesencephalic cultures, as shown by increases in cellular viability and [^{3}H]DA uptake [43]. Similarly, EGCG was reported to protect human neuroblastoma cells from damage induced by 6-OHDA and MPP^+ [44]. EGCG also protects primary hippocampal neurons [45] and rescues rat pheochromocytoma (PC12) cells from amyloid-β peptide (Aβ)-induced toxicity [46]. More recently, EGCG was reported to exert a neurorescue activity in long-term serum-deprived PC12 cells and to promote neurite outgrowth, as manifested by the expression of a surrogate marker of cell differentiation, growth-associated protein GAP-43 (GAP-43) [47]. This could have important implications with regard to aging, PD and AD, suggesting a potential therapeutic use of EGCG in regenerating injured neuronal cells.

Neuroprotection and Neurotoxicity by Low and High Concentrations of Catechins and Other Flavonoids

Studies from our and other laboratories have shown that green tea polyphenols display a concentration-dependent window of neuroprotective action: They protect

at low micromolar concentrations, whereas they become pro-oxidant and pro-apoptotic when increasing the concentrations over 10–20 μ*M* [44, 48]. This bell-shaped pattern is typical of antioxidative drugs, such as vitamin C [49], R-apomorphine [50] and DA [51], being neuroprotective at low (1–10 μ*M*) concentrations, while having pro-oxidant/pro-toxic activity at higher (10–50 μ*M*) concentrations. The toxicity of not only green tea polyphenols, but also of several other flavonoids, is responsible for their antiproliferative and chemopreventive actions. The anticancer properties of polyphenols is attributed to the ability to inhibit phase I and induce phase II carcinogen metabolizing enzymes (in animals) that initiate carcinogenesis, inhibition of cell cycle progression effectors, promotion of ROS and nitrogen species (thereby collapsing the mitochondrial membrane potential), induction of p53 and apoptogenic factors and inactivation of protein kinases that contribute to survival-associated signal transduction [for extensive reviews see 5, 52, 53]. Since the present article applies to the mechanisms of neuroprotection by tea catechins, the literature regarding their anticarcinogenic or pro-apoptotic properties will not be reviewed.

Mechanism of Neuroprotection by Green Tea Catechins

Cell Signaling Pathways

Selective Activation of Protein Kinase C (PKC) in Brain Neurons

PKC expression has been previously coupled with the preservation of cell survival and the formation and consolidation of different types of memory [54–56]. The induction of PKC activity in neurons is thought to be a prerequisite for neuroprotection against several exogenous insults. Indeed, PKCε activation after ischemic preconditioning or pharmacologic preconditioning (with PKCε, NMDA, or A1AR agonists) was shown essential for neuroprotection against oxygen/glucose deprivation in organotypic slice cultures [57]. In accordance, activation of PKC by estrogen or by the grape flavonoid resveratrol, in rat cortical or hippocampal neurons, respectively, protects against Aβ toxicity [58, 59]. Also, we have recently shown that the anti-Parkinson/monoamine oxidase-B (MAO-B) inhibitor drug, rasagiline (Teva Pharmaceutical Industries) [60], prevented PC12 cell death induced by serum deprivation via PKC signaling cascade [61]. Similarly, we have reported that phosphorylative activation of PKC by EGCG is responsible for the protective effects against 6-OHDA- and Aβ-induced cell death in SH-SY5Y and PC12 cells [44, 46] and for the neurorescue effect against long-term growth factors withdrawal in PC12 cells [47]. This is supported by the observation that EGCG could not overcome cell death under PKC pathway blockade, as determined both morphologically and by monitoring various apoptotic markers, suggesting that this cascade is essential for the neuronal protection and rescue effects of EGCG. Consistent with these findings, recent animal studies have shown that two weeks consumption of EGCG (2 mg/kg) led to a highly significant up-regulation of PKCα isoform in mice striatum [62] and to a significant increase in PKC isoenzymes α and ε in the membrane and cytosolic fractions of mice hippocampus [46]. The implication of PKCα in neuronal survival by EGCG is further demonstrated in vitro by the rapid translocation of PKCα to the membrane compartment in PC12 cells, in response to EGCG (fig. 1). PKCα is a well-established neuron cell survival factor participating also in cell growth and differentiation [63, 64]. In support, a recent report shows that treatment of human cells with EGCG induces a specific translocation of PKCα to the membrane [65].

More direct evidence implicating PKC in EGCG mechanistic action has come from a recent study employing solid-state nuclear magnetic resonance, showing that EGCG interacts with the head group region of the phospholipids within lipid bilayers from liposomes [66]. The interaction pattern of EGCG in terms of rotational motion within the lipid bilayers was similar to that described for 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [67], a phorbol ester. Phorbol esters are prototype activators of PKC, suggesting that direct interaction of green tea catechins with cell membranes may be sufficient for the rapid activation of PKC by EGCG previously reported by us [44]. The impact of EGCG on membrane fluidity may give rise to activation of other membrane-associated signaling pathways (e.g. G proteins), which can contribute as well to its protective action. This clearly needs to be examined.

Modulation of Other Signal Transduction Pathways and Intracellular Transducers

In addition to PKC, other cell signaling pathways have been also implicated in the action of green tea catechins such as the mitogen-activated protein kinases (MAPKs) and phosphatidylinositide 3′-OH kinase (PI3K)/AKT signaling cascades. These cascades have been shown to play central functions in neuronal protection against a variety of extracellular insults and to be essential for neu-

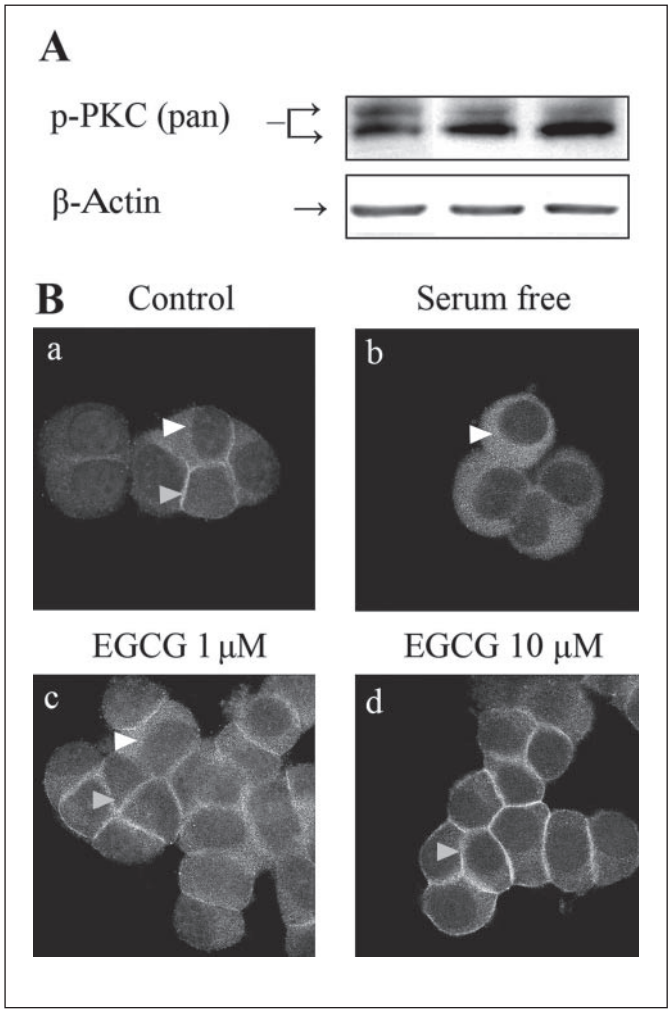

Fig. 1. EGCG activates p-PKC (pan) and induces a rapid activation of PKCα and translocation to the membrane. **A** Cell lysates from PC12 cells deprived of serum for 24 h before short-term (15 min) exposure to EGCG (1–10 μ*M*) were subjected to SDS-PAGE and Western blot, with p-PKC (pan) antibody. **B** Cultured PC12 cells grown with full serum (FS) support (control) were deprived of serum (SF) for 24 h before short-term (15 min) exposure to EGCG (1–10 μ*M*). The cells were fixed and permeabilized for subcellular localization of PKCα by confocal microscopy, using an isoenzyme-specific antibody and FITC-conjugated secondary antibody (light areas). DRAQ5 stains nuclei (dark areas). Under FS conditions (control) PKCα is evenly confined to both plasma membrane (grey arrow) and cytosol (white arrow) (**a**). Upon serum withdrawal, PKCα immunostaining is mosly cytosolic (**b**). In cells treated with EGCG PKCα is, in its majority (1 μ*M*), or entirely (10 μ*M*) localized to the cell membrane (arrows) (**c**, **d**). The images are representative fields from 3 independent experiments, all showing the same results. Taken from Reznichenko et al. [47].

ronal differentiation and survival [68–70]. OS seems to be a major stimulus for MAPK cascade, which might lead to cell survival/cell death [for review see 71]. Among the MAPKs the extracellular signal-regulated kinases (ERK1/2) are mainly activated by mitogen and growth factors [72], while p38 and c-jun-N-terminal kinase (JNK) respond to stress stimuli [73]. However, there have been reports where activation of ERK1/2 is thought to mediate neuronal injury such as in focal ischemia [74], in glutamate and oxidized-low-density-lipoprotein-induced toxicity [40, 75] and in cytotoxicity and activation of caspase-3 in the extraneuronal hepatoma HepG2 [76] and HeLa [77] cell lines, respectively. Increasing evidence shows that catechins can protect against neuronal cell death caused by exogenous OS-inducing agents through modulation of ERK activity [40, 44]. In this regard, a number of flavonoids and phenolic antioxidants, at their respective low protective concentrations, were demonstrated to activate the expression of some stress-response genes, such as phase II drug-metabolizing enzymes, glutathione-s-transferase and heme-oxygenase1 [77], likely via activation of the MAPK pathway [78].

More recently, additional signaling pathways, including PI3K/AKT, protein kinase A (PKA) and calcium, have been implicated in the neuroprotective action of catechin flavonoids. These studies have been conducted mainly in extra-neuronal tissue such as skin and heart. For example, topical application of EGCG induces proliferation of human normal epidermal keratinocytes through stimulation of ERK1/2 and AKT [79]. Other investigators reported a rapid activation of endothelial nitric oxide synthase after EGCG treatment by a process that involves PI3K, PKA and AKT in endothelial cells [80] and a decrease in iNOS expression via inactivation of STAT-1alpha in epithelial and colon cell lines [81]. Consistent with this, Townsend et al. [38] have recently reported that in cardiac myocytes EGCG protects against ischemia/reperfusion-induced apoptosis through a mechanism involving reduction of STAT-1 phosphorylation (inactivation) and of his downstream pro-apoptotic target gene, Fas. The discrepancy or divergence in the different signal pathway activation by EGCG may reflect differences in cell tissue (e.g. neuronal vs. peripheral), or in the downstream pathways being under the control of the different kinases, which may diverge into different responses, thereby providing cell function diversity.

Anti-Apoptotic Activity

EGCG has been reported to exert a biphasic mode of action as a function of concentration. The low micromo-

lar concentrations are responsible for the anti-apoptotic/neuroprotective actions of EGCG. This receives further recognition from an experiment employing customized cDNA microarray designed to clarify the molecular mechanisms involved in the cell survival action of EGCG [44]. The results revealed that a low EGCG concentration decreased the expression of pro-apoptotic genes *bax, bad, caspases-1* and *6*, cyclin-dependent kinase inhibitor *p21*, cell-cycle inhibitor *gadd45*, *fas-ligand* and tumor necrosis factor-related apoptosis-inducing ligand *TRAIL*, in SH-SY5Y neuronal cells. The same group has reported recently that EGCG reduced the expression of several apoptogenic factors when given after long-term serum deprivation of PC12 cells [47]. These findings are supported by an in vivo study showing that two weeks oral consumption of EGCG (2 mg/kg) alone caused a complete disappearance of Bax immunoreactivity, specifically in the dopaminergic neurons of the SNpc [62] and counteracted the robust increase of Bax protein when administered before MPTP intoxication in the same area.

Bax alone, or in conjunction with the BH-3-only proteins (e.g. Bad, Bid, Bim, Noxa, Puma), can trigger the opening of the mitochondrial megachannel permeability transition pore (mPTP), or a specific channel in the outer mitochondrial membrane, both of which promote the fall in mitochondrial membrane potential, leading to cytochrome *c* release and consequent cell death [82, 83]. The decline in Bax expression by EGCG may favor the increase in the ratio of Bcl-2/Bcl-xL to Bax/Bad proteins, thereby contributing to mitochondrial stability and regulation of mPTP [84]. Protection of mitochondrial integrity is of major importance, especially in the case of post-mitotic cells such as neurons and heart muscle cells, which are commonly not renewed. Thus, it is not surprising that one of the major neuroprotective strategies in PD, AD and other neurodegenerative diseases, where increased OS, perturbed cellular energy and ion homeostasis have been implicated, includes pharmacological agents directed to specific mitochondrial targets. In this respect, *Ginkgo biloba* extract EGb 761 or its individual components were shown to protect mitochondria integrity by protecting against uncoupling of oxidative phosphorylation, thereby increasing ATP levels and by increasing the expression of the mitochondrial DNA-encoded COX III subunit of cytochrome oxidase [for review, see 85]. Flavonoids may also affect mitochondrial integrity by increasing GSH levels and preventing the influx of calcium, as previously reported [86, 87].

Metal-Chelating Activity

One cardinal feature of neurodegenerative diseases including AD, PD, Huntington's disease, ALS, Friedreich's ataxia, multiple sclerosis, and aceruloplasminemia is the appearance of excessive iron at degenerative neuronal sites [17]. The buildup of an iron gradient in conjunction with ROS (superoxide, hydroxyl radical and nitric oxide) are thought to constitute a major trigger in the demise in all theses diseases. Therefore, the chelation of free cellular ferric and ferrous ions by the different metal chelators make them potential agents to combat iron-induced generation of reactive oxygen radicals (by the Fenton and Haber-Weiss reactions) and aggregation of alpha (α)-synuclein and Aβ in PD and AD, respectively.

Iron Chelation for AD

A significant body of evidence point to an 'amyloid cascade' event in the pathogenesis of AD, where amyloid precursor protein (APP) is processed to Aβ, by β- and γ-secretases, which spontaneously self-aggregate in the presence of divalent metals (Fe^{2+}, Cu^{2+}) into neurotoxic amyloid fibrils in the neocortex [88]. Iron was shown to promote both deposition of Aβ and OS, which is associated with the plaques [89]. In addition, iron has taken center stage in AD as a consequence of the studies by Rogers and coworkers [90] who described the existence of an iron responsive element (IRE-type II) in the 5′UTR region of APP mRNA. APP is post-transcriptionally regulated by iron regulatory proteins (IRPs), which are labile iron pool-sensitive cytosolic RNA proteins binding specifically to the IRE located in the 5′ or 3′ untranslated regions of iron metabolism-associated mRNAs. Changes in iron status (iron overload or depletion) lead to compensating changes in the IRP/IRE system of translational control of iron homeostasis. For example, the APP 5′-UTR conferred translation was selectively down-regulated upon intracellular iron chelation, in a similar manner as the iron-storage protein ferritin, which also possesses an IRE in its 5′-UTR mRNA [90].

Iron removal has effects suggestive of inhibition of key enzymes or other metalloproteins, for instance in mimicking hypoxia, whereas many of the effects of iron overload may be the result of signaling associated with OS. Hypoxia and iron chelation have similar effects on genes regulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α), a master regulator orchestrating the coordinated induction of an array of hypoxia-sensitive genes [91]. The target genes of HIF are especially related to angiogenesis, cell proliferation/survival and glucose/

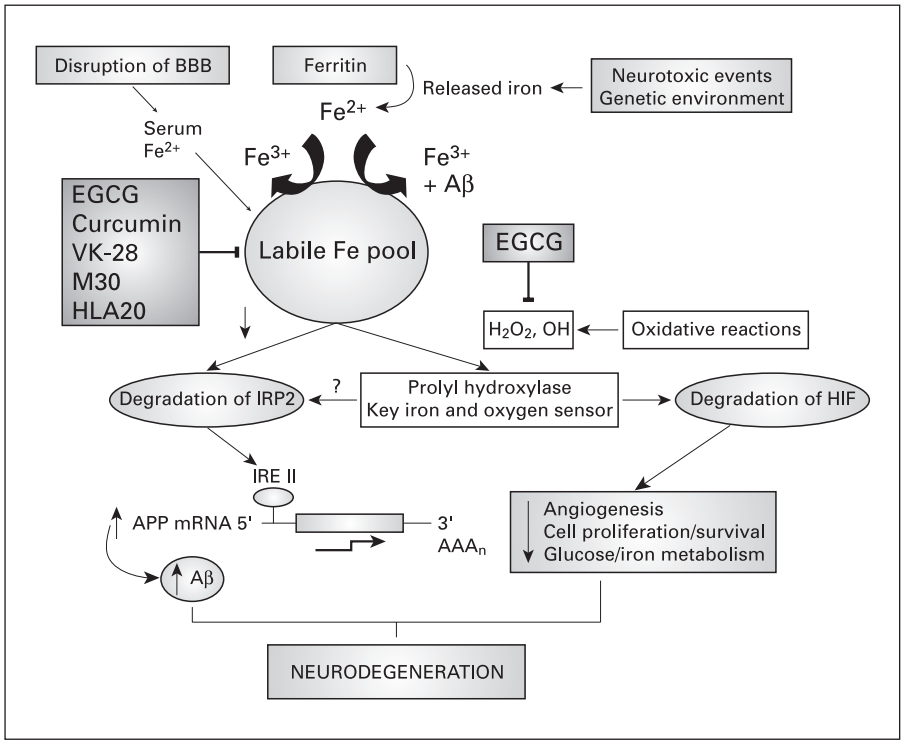

Fig. 2. Iron-induced neurodegeneration in AD via transcriptional activation of APP mRNA. Neurodegeneration can result from abnormal serum iron transport to the neurons because a disruption in the blood-brain barrier (BBB) or from release from its storage protein ferritin, thereby increasing the free-labile iron pool (ionic iron). Labile iron can increase the production of amyloid precursor protein (APP) by down-regulating the activities of iron regulatory proteins (IRP1 and IRP2, inactivation and proteasomal degradation, respectively), thereby promoting the translation of APP mRNA from its 5′-UTR type II. Ionic iron may also cause aggregation of amyloid-β peptide (Aβ) to form toxic aggregates, which, in turn, can initiate $OH^{\bullet}$ generation, causing oxidative stress (OS). Increased iron and OS may activate the prolyl hydroxylase enzymes which are key iron and oxygen sensors, leading to proteasomal-mediated degradation of the transcription factor hypoxia-inducible factor 1α, a master regulator orchestrating the coordinated induction of a wide array of survival genes. It has been also suggested that IRP2 can be a substrate for prolyl hydroxylase. Neuroprotective agents that can be used to prevent iron-induced neurodegeneration include M30 and HLA20 (bifunctional iron chelator-MAO inhibitors), VK-28, EGCG and curcumin (iron chelators). For a more detailed explanation, read text.

iron metabolism [92]. The mechanism of HIF-1α activation by iron chelation is not well understood. Fe(II)/2-oxoglutarate-dependent dioxygenases have been identified that hydroxylate critical proline and asparagine residues in HIF and upon high oxygen levels and iron overload, target HIF for degradation [93]. Thus, these prolyl hydroxylase enzymes act as key iron and oxygen sensors. This may explain the decrease in cell survival genes described in neurodegenerative diseases such as phosphofructokinase and the angiogenic factor VEGF, both regulated by the HIF proteins [94]. Interestingly, the free iron-induced proteasomal-mediated degradation of IRP2 involves also activation a prolyl hydroxylase and is inhibited by iron chelators [95, 96]. Thus, it is possible that IRP2 is a substrate for this enzyme, in a similar way as HIF, signaling it for protein degradation. This suggests a convergence of both iron and OS to a common pathway triggering the neurotoxic degenerative cascade (for a detailed explanation, see fig. 2).

The involvement of metals in the plaques of AD patients and the presence of an IRE in the unstranslated region of APP mRNA, have encouraged the development of iron chelators as a major new therapeutic strategy for the treatment of AD. In fact, intramuscular administration of the prototype iron chelator desferrioxamine (DFO) slowed the clinical progression of AD dementia [97], and some success has also been achieved with another metal-complexing agent, clioquinol [98]. However, clioquinol is highly toxic [99] and DFO has poor penetration across the blood-brain barrier [17].

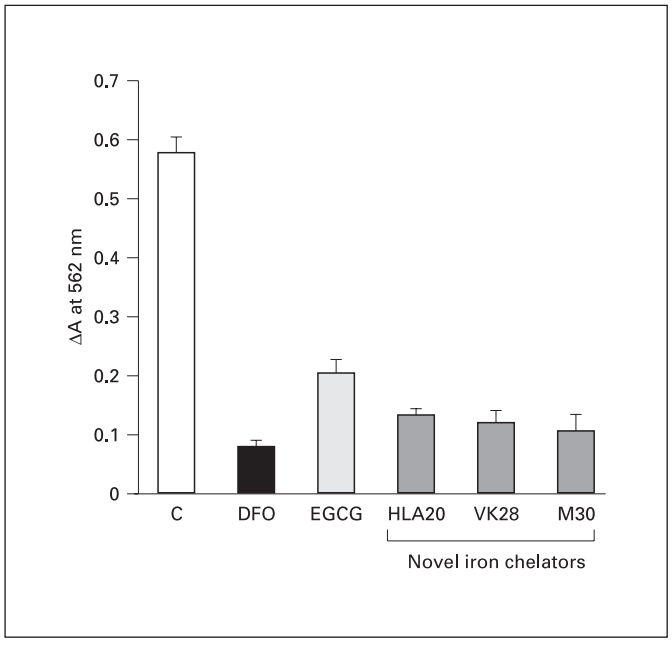

Fig. 3. Comparison of the Fe^{2+}-chelating potency of EGCG to other iron chelators. The metal-binding capacity of EGCG was compared to that of DFO and the novel iron chelators VK28, HLA20 and M30, by assessing their ability to compete with ferrozine for the ferrous ions, resulting in decrease in the absorbance at 562 nm. Ferrozine can quantitatively react with Fe^{2+} to form Fe^{2+}-ferrozine complex with a strong absorbance at 562 nm. In the presence of other chelating agents, the complex formation is disrupted with the result that the absorbance at 562 nm is decreased. 0.1 m*M* of drug was mixed with 0.1 m*M* ferrozine in 5% ammonium acetate (pH 7) followed by the addition of 0.02 m*M* $FeSO_4$. After 2 h incubation, the absorbance (at 562 nm) of resulting solutions was read. Considering that the purpose of this assay was to evaluate the ability of drugs to compete with the iron indicator ferrozine, drugs and ferrozine were used at equal concentrations. Chelating effect of drug on Fe^{2+} was calculated as follows: Chelating effect (%) = [1–(absorbance of sample at 562 nm)/(absorbance of control at 562 nm)] × 100. The order of chelating potency for complexing Fe^{2+} in solution is Desferal (DFO) > M30 ≥ VK28 ≥ HLA20 > EGCG.

A possible novel promising therapeutic approach for treating AD, PD and ALS with non-toxic, brain permeable metal chelators could be the use of the naturally occurring polyphenols, such as EGCG and curcumin, which by being of natural origin may not exert toxic side effects inherent to synthetic drugs. Both compounds have well-characterized antioxidant, metal (iron and copper) chelating and anti-inflammatory activities [12, 100] and have been demonstrated to exert neuroprotective activity against a variety of neurotoxic insults, as well as to regulate APP processing and Aβ burden in cell culture and in vivo [12, 101]. Our recent studies have shown that prolonged administration of EGCG to mice induced a reduction in holoAPP levels in the hippocampus [46]. Indeed, this effect may be related to the iron-chelating properties of EGCG, leading to a decrease in the free-iron pool. This in turn results in the suppression of APP mRNA translation by targeting the IRE-II sequences in the APP 5′-UTR [90], as was recently shown for DFO and the bifunctional amyloid-binding/metal-chelating drug XH1 [102] (fig. 2).

The concept of metal chelation as a neuroprotective strategy has led us to the development of non-toxic, lipophilic, brain-permeable iron chelators for progressive neurodegenerative diseases [103]. Compounds such as VK-28 (Varinel) [104] and the multifunctional iron chelators HLA20 and M30 [105, 106], which possess the propargylamine MAO inhibitory and neuroprotective moiety of rasagiline, display good cell permeability and are protective against 6-OHDA toxicity in differentiated P19 cells [107]. Comparative analysis of the Fe^{2+}-chelating potency of EGCG, the prototype DFO and other pharmacological iron chelators, has revealed similar binding potency (fig. 3). Both EGCG and the iron chelator M30 were shown by us to induce a significant down-regulation of membrane-associated holoAPP level in the mouse hippocampus (fig. 4), SH-SY5Y and CHO cells expressing the APP 'Swedish' mutation (data not shown). This may have a direct influence on Aβ levels and plaque formation, as shown in preliminary studies for XH1 [102]. Indeed, using a nucleation-dependent polymerization model, it has been shown that wine and green tea polyphenols are able to inhibit formation, extension and destabilization of β-amyloid fibrils [108].

Other potential beneficial effect of EGCG in AD may be related to our previous studies demonstrating the ability of EGCG to promote the non-amyloidogenic pathway, via a PKC-dependent activation of α-secretase, thereby increasing sAPPα [46]. sAPPα has been demonstrated to posses potent neuroprotective activities against excitotoxic and oxidative insults in various cellular models [109] and it was shown to protect against p53-mediated apoptosis [110]. Moreover, sAPPα promotes neurite outgrowth [111], regulates synaptogenesis [112] and exerts trophic effects on cerebral neurons in culture. As sAPPα and Aβ are formed by two mutually exclusive mechanisms, stimulation of the secretory processing of sAPPα might prevent the formation of the amyloidogenic Aβ. Thus, EGCG may influence Aβ levels, either via translational inhibition of APP or by regulating APP processing. Finally, iron chelation by EGCG may ablate Fe^{3+}-induced aggregation of hyperphosphorylated tau (PHFτ), the major constituent

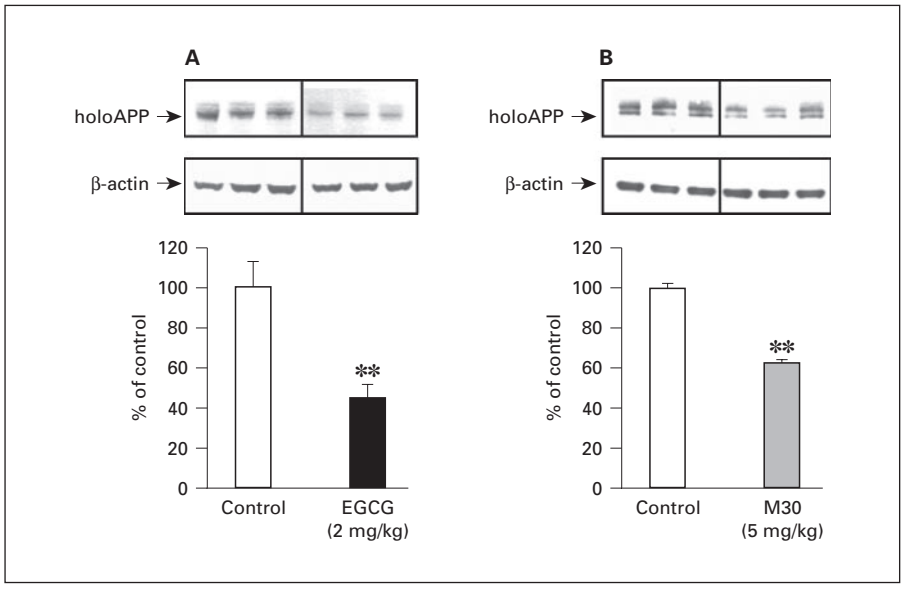

Fig. 4. Effect of EGCG and M30 on APP processing in mice hippocampus. Representative Western blots of levels of holoAPP in the membrane compartment, obtained from hippocampus of mice treated with EGCG (2 mg/kg) (**A**), or with M30 (5 mg/kg) (**B**) for 14 days detected with 22C11 antibody (directed to the to APP N-terminus) or with a C-terminus APP antibody, respectively. Densitometric analysis is expressed as percent of the control, untreated animals after normalizing to the levels of β-actin. Data are expressed as the mean ± SEM (n = 6 mice in each group). ** $p < 0.03$ vs. control. Figure drawn using information from Levites et al. [46].

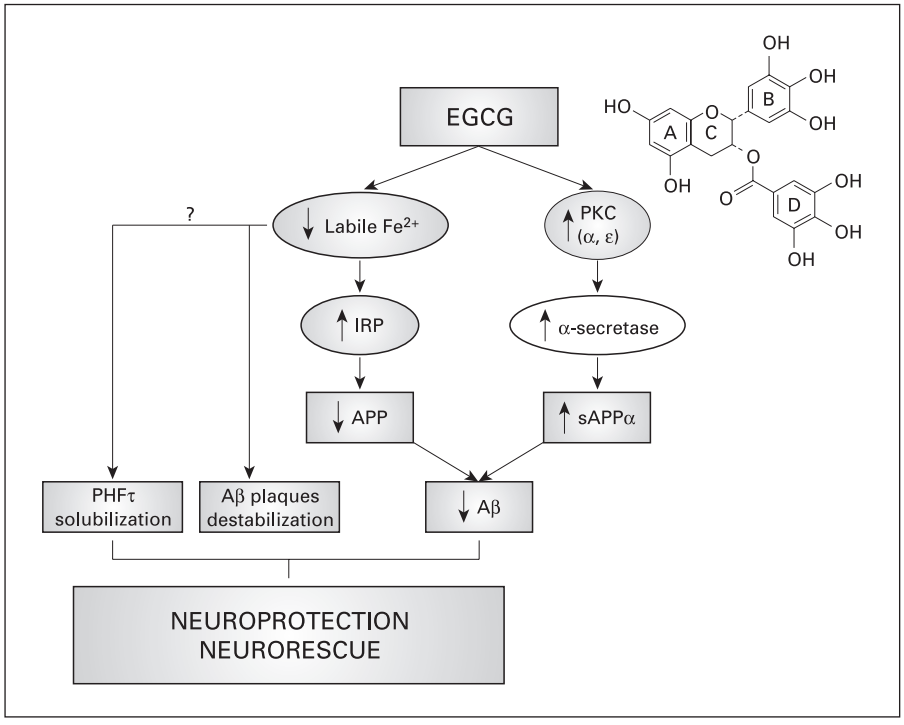

Fig. 5. Proposed schematic model for EGCG neuroprotective effect via regulation of APP processing and Aβ formation. ↑ Increased levels/activity, ↓ decreased levels/activity. For full explanation, see text.

of neurofibrillary tangles in AD brains [113] (a descriptive explanation is depicted in fig. 5).

Iron Chelation for PD

Numerous studies have shown that there is a progressive accumulation of iron and ferritin in the SN pars compacta (pc) of PD patients [17, 27, 114]. Specifically, redox-active iron has been observed in the rim of Lewy body, the morphological hallmark of PD, also composed of lipids, aggregated α-synuclein (concentrating in its peripheral halo) and ubiquitinated, hyperphosphorylated neurofilament proteins [115]. α-Synuclein associated

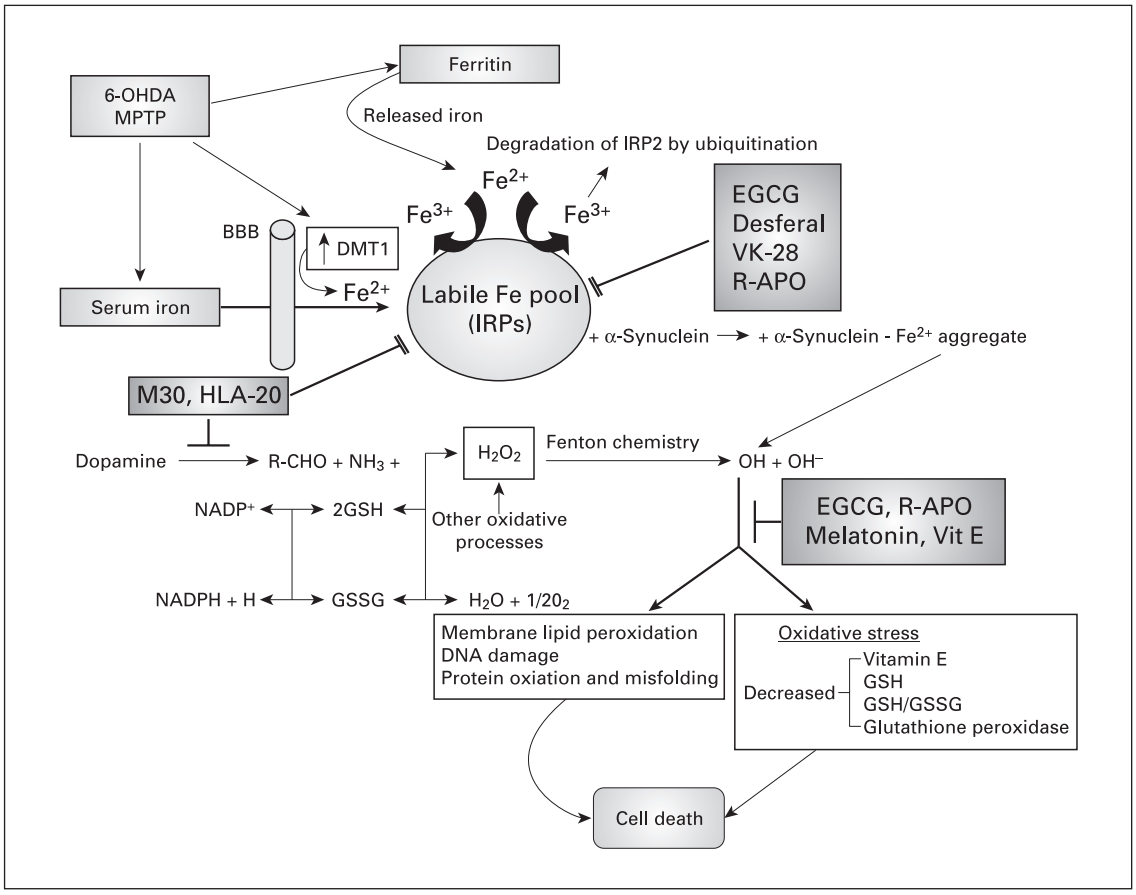

Fig. 6. Possible mechanism of neurotoxin-induced iron uptake, release and interaction with α-synuclein resulting in OS initiated neurodegeneration and its prevention by iron-chelating/antioxidants. The mechanism by which 6-OHDA and MPTP induce increase of iron in substantia nigra pars compacta and within the melanin-containing neurons is not known. These neurotoxins may (a) activate the divalent metal transporter 1 (DMT1) which is responsible for iron transport into the brain across the cell membrane; (b) alter the blood-brain barrier (BBB), thereby allowing iron access to the brain; (c) induce release of iron from ferritin which enters the labile (redox-active) pool of iron. It is the labile pool of iron which can initiate the Fenton chemistry in response to the presence of hydrogen peroxide, thus generating the highly reactive hydroxyl radical ($OH^{\bullet}$). The resultant effect is the depletion of cell-reduced glutathione (GHS), the rate-limiting cofactor of glutathione peroxidase, the main enzymatic pathway in the brain, to eliminate hydrogen peroxide. Labile pool of iron can also cause aggregation of α-synuclein to the neurotoxic form, which can also generate $OH^{\bullet}$. The net effect is oxidative-stress-dependent damage to neuron antioxidant mechanism, membrane lipid peroxidation, demise of cell and mitochondrial membrane, protein misfolding and ultimate cell death. Neuroprotective agents that can be used to prevent iron-induced neurodegeneration include M30 and HLA20 (bifunctional iron chelator-MAO inhibitors); desferal, VK-28, R-APO (R-apomorphine) and EGCG (iron chelators); R-APO, EGCG, melatonin and Vit E (vitamin E) (radical scavengers). Sharp arrows indicate positive inputs, whereas blunt arrows are for inhibitory inputs. Reproduced, with minor modifications, from Youdim et al. [106].

with presynaptic membrane is not toxic; however, a number of recent studies [116–118] have shown that it forms toxic aggregates in the presence of iron and this is considered to contribute to the formation of Lewy body via OS, being one of its constituents.

Our recent high throughput gene expression study in the SNpc of Parkinsonian brains employing Affymetrix chip technology [29] has revealed a significant increase on the key iron and oxygen sensor EGLN1 gene coding for an isoform of 2-oxoglutarate-dependent dioxygenase hydroxylase (see previous section). Excessive production of EGLN1 hydroxylase in the SNpc may lead to a fall in IRP2 and subsequent decrease in transferrin receptor (TfR) mRNA and increase in ferritin levels, both subjected to positive and negative transcriptional regulation by IRP2, respectively [119, 120]. Recent studies in knock-

out mice for IRP2 have revealed accumulation of iron in the striatum with substantial bradykinesia and tremor [121].

Consistent with the pivotal role of iron in neurodegeneration is the finding that the iron chelator desferal prevents cytochrome c-induced-α-synuclein aggregation and toxicity in vitro [122] and attenuates dopaminergic neurotoxicity in response to neurotoxins MPTP and 6-OHDA in vivo [123, 124]. In line with the iron-chelating feature of EGCG, this polyphenol was shown to prevent α-synuclein accumulation and to attenuate IRP2 depletion, in the SNpc of mice intoxicated with MPTP, when given orally for a period of 2 weeks [62]. This may be associated to our previous studies where green tea extract or EGCG prevented DA-containing neuron degeneration and tyrosine hydroxylase activity decrease [30]. In spite of the absence of clinical trials regarding tea polyphenols and PD, epidemiological studies have shown reduced risk of PD associated with consumption of 2 cups/day or more of tea [125] and a much lower prevalence of PD in Chinese population than in white people [126, 127].

Additional studies examining the effect of iron chelation, by either transgenic expression of the iron-binding protein, ferritin, or oral administration of the metal chelator clioquinol, have shown significant attenuation of MPTP-induced neurotoxicity [128]. These findings have now been substantiated with systemic injection of the brain-permeable iron chelator, VK-28, to rats in response to 6-OHDA [104]. In line, nutritional iron deficiency protects rats against kainate and 6-OHDA [129]. Figure 6 summarizes the mechanism of neurotoxin-induced iron uptake, release and interaction with α-synuclein resulting in OS initiated neurodegeneration and its prevention by iron-chelating/antioxidant agents.

Conclusions

It is likely that syndromes such as AD and PD will require multiple- drug therapy to address the varied pathological aspects of the disease. Therefore, the use of compounds with poly-pharmacological activities or cocktail of drugs is a promising therapeutic approach for the treatment of neurodegenerative diseases. Indeed, a wealth of new data indicates that green tea catechins are being recognized as multifunctional compounds for neuroprotection. They act as radical scavengers, iron chelators and modulators of pro-survival genes, and PKC signaling pathway. The use of EGCG as a natural, non-toxic, lipophilic brain permeable neuroprotective drug is advocated for 'ironing out iron' from those brain areas where it preferentially accumulates in neurodegenerative diseases [106]. Thus, green tea catechins may have potential disease-modifying action. Future efforts in the understanding of the protective mechanism of action of these polyphenols should concentrate on deciphering the cellular targets affected by these compounds and other neuroprotectants.

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