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Molecular mechanisms of the neuroprotective/neurorescue action of multi-target green tea polyphenols

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1. ABSTRACT

Mounting evidence suggests that lifestyle factors, especially nutrition are essential factor for healthy ageing. However, as a result of the increase in life expectance, neurodegenerative diseases like Alzheimer's and Parkinson's (AD and PD, respectively) are becoming an increasing burden, as aging is their main risk factor. Brain aging and neurodegenerative diseases of the elderly are characterized by oxidative damage, dysregulation of redox metals homeostasis and inflammation. Thus, it is not surprising that a large amount of drugs/agents in therapeutic use for these conditions are antioxidants/metal complexing, bioenergetic and anti-inflammatory agents. Natural plant polyphenols (flavonoids and non-flavonoids) are the most abundant antioxidants in the diet and as such, are ideal nutraceuticals for neutralizing stress-induced free radicals and inflammation. Human epidemiological and new animal data suggest that green and black flavonoids named catechins, may help protecting the aging brain and reduce the incidence of dementia, AD and PD. This review will present salient features of the beneficial multi-pharmacological actions of black and green tea polyphenols in aging and neurodegeneration, and speculate on their potential in drug combination to target distinct pathologies as a therapeutic disease modification approach.

2. INTRODUCTION

Green tea is made from the dried leaves of Camellia sinensis, a small plant grown mainly in China, Japan and Southeast Asia and is consumed in the form of green tea, oolong tea or black tea, all brewed from Camellia sinensis (1). Standing after water, tea signifies the second most frequently consumed beverage worldwide, which varies its status from a simple ancient drink and a cultural tradition to a nutrient endowed with possible neurobiological-pharmacological actions beneficial to human health. The medicinal properties of green tea extract had been attributed to its high content of polyphenolic flavonoids known as catechins. Flavonoids are natural antioxidant substances present in dietary sources from fruits and vegetables and from plant-derived beverages such as pomegranate juice, raspberry, blueberries, red wine and tea. Flavonoids include the subclasses of flavones, isoflavones, flavanols, flavans and flavonols. Catechins are flavanols that are especially concentrated in green tea and account for 30-40% of the dry weight of the leaves (2, 3). Green tea is much richer in catechins than other beverages and compared to black tea, it contains around four times more of the catechin fraction (4). Among the tea catechins, (–)-epigallocatechin-3-gallate (EGCG) is the major constituent, accounting for more than 10% of the extract

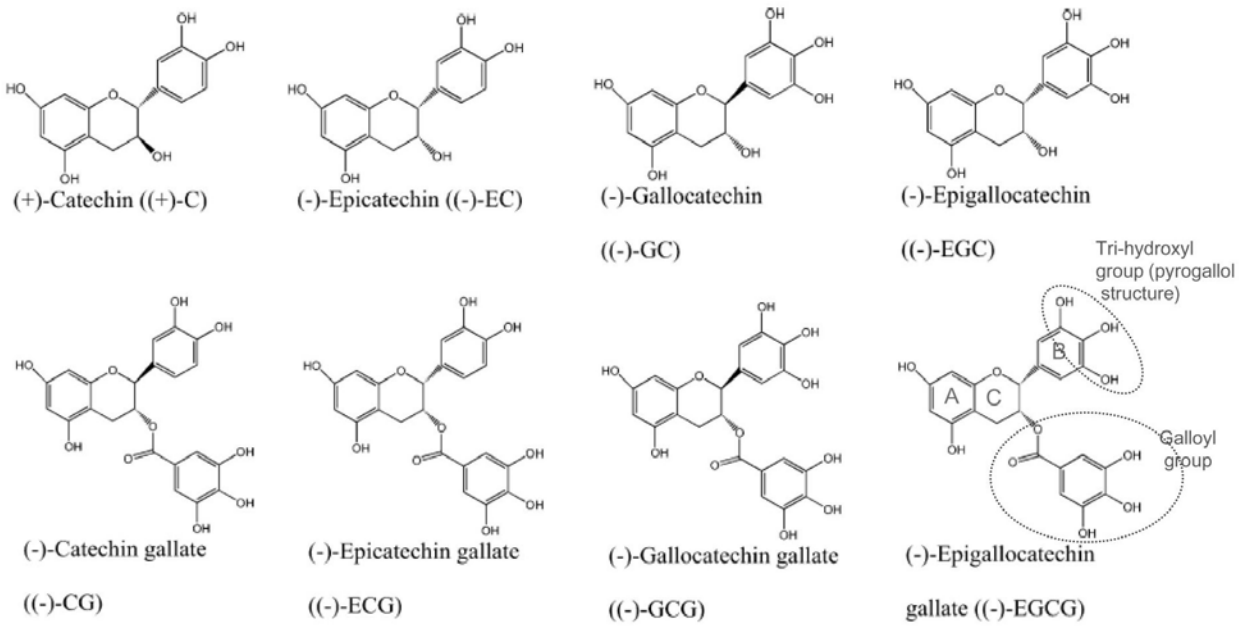

Figure 1. Molecular structure of the main catechins (flavanols) in green tea extract

dry weight; several other polyphenolic compounds found in lower abundance in green tea include (–)-epigallocatechin, (EGC) > (–)-epicatechin (EC) ≥ (–)-epicatechin-3-gallate (ECG) (Figure 1). All four tea catechins have been demonstrated to be potent antioxidants resulting from their direct scavenging of reactive oxygen and nitrogen species (ROS and NOS, respectively), induction of endogenous antioxidant enzymes and the capacity to bind and chelate excess of divalent metals, such as iron and copper (5-7).

Owed to these properties, green tea extract or/and its isolated catechins were tested in a spectrum of neuronal cultures and animal models of neurodegenerative diseases and aging and demonstrated to protect and rescue brain neurons against various exogenous damages (see next sections). Systematic mechanistic studies revealed that the protective contribution of tea catechins relied not only on their known antioxidant/metal chelation activities, but also to their ability to modulate several signal transduction pathways, cell survival/death genes, and mitochondrial function (8, 9). These properties and the reported brain penetrance of some catechins including EGCG (10-12), confer them a multi-target potential to address some of the varied pathologies in aging and neurodegenerative diseases. These include, among others, oxidative stress (OS) damage, dysregulation of redox metals homeostasis, impairment of protein handling and aggregation associated with dysfunction of the ubiquitin-proteasome system (UPS), depletion of endogenous antioxidants, reduced expression of trophic factors, glutamatergic excitotoxicity and inflammation (13-19).

The following sections will elaborate on epidemiological and clinical evidence on the beneficial action of tea flavonoids in aging and neurodegeneration, describe their suggested mechanisms of neuroprotection by simultaneous manipulation of multiple central nervous system targets and discuss a scenario concerning their potential, in drug combination to target distinct pathologies.

3. EPIDEMIOLOGICAL AND CLINICAL STUDIES WITH BLACK AND GREEN TEA IN ALZHEIMER'S AND PARKINSON'S DISEASES

Multiple lines of evidence, mostly from preclinical and epidemiological studies suggest that green tea consumption is associated with reduced risk of severe human malignancies such as cancer, cardiovascular diseases and diabetes, which have been linked to the antioxidant/pro-oxidant properties of its polyphenol constituents (Figure 2) (20-25). In spite of the lack of systematic clinical trials with tea polyphenols in neurodegenerative diseases, the consumption of tea has been suggested to help in supporting the brain as people get older, owing to its high flavonoid (catechin) content. Drinking tea has been found inversely correlated with the incidence of dementia, Alzheimer's disease and Parkinson's disease (AD and PD, respectively). Epidemiological data in elderly Japanese subjects, found that higher consumption of green tea, but not black tea, is associated with lower prevalence of cognitive impairment (26). A recent cross-sectional analysis of participants from the Singapore Longitudinal Ageing Study revealed that regular consumption of black or oolong tea was associated with lower risks of cognitive impairment and decline (27). In the Chinese nonagenarians/centenarians study with 681 participants (67.25% women), men with cognitive impairment had significantly lower prevalence of habits of tea consumption, but significantly higher prevalence of habits of smoking and alcohol consumption (28). Similar outcomes have been found in PD. In the USA, people that consumed 2 cups/day or more of tea presented a decreased

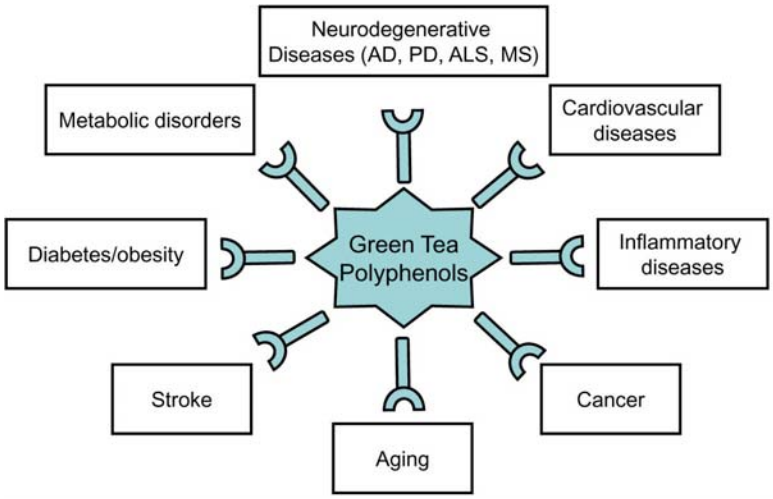

Figure 2. Multiple pharmacological activities of green tea catechins. AD (Alzheimer's disease), PD (Parkinson's disease), ALS (Amyotrophic lateral Sclerosis), MS (Multiple Sclerosis).

risk of PD (29) and a prospective 13-years study of nearly 30,000 finnish adults, demonstrated that drinking 3 or more cups of tea per day is associated with a reduced risk of PD (30). In ~63,000 Asian participants from the prospective Singapore Chinese Health Study followed for 12 years, an inverse association between black tea and PD was found, independent of smoking or caffeine consumption (31). A recent retrospective study with 278 consecutive PD patients reported that consumption of more than 3 cups of tea per day delayed age of motor symptoms onset by 7.7 years and also, smoking ≥10 cigarette packs a year delayed age of PD onset by 3.2 years (32). Regarding clinical studies, a first multicenter, double-blind, randomized, placebo-controlled delayed study to evaluate the safety, tolerability and efficacy of green tea polyphenols in slowing disease progression in patients with early PD, was conducted by the Chinese Parkinson Study Group and supported by The Michael J. Fox Foundation. 410 untreated people with PD were enrolled at 32 Chinese Parkinson Study Group sites. In the first phase of the study participants were randomized to 0.4 g (102 people), 0.8 g (103 people), or 1.2 g (104 people) of green tea polyphenols daily, or placebo (101 people). At 6 months, the placebo group switched to 1.2 g of green tea polyphenols daily for 6 more months (phase 2). If persistent differences are observed between the two groups at the end of phase 2, they cannot be readily explained by effects on symptoms alone, since both groups are receiving the same treatment. Thus, these differences are consistent with the possibility of a disease-modifying effect. A similar rationale has been recently implemented in the ADAGIO (Attenuation of Disease progression with Azilect® Given Once-daily) trial with the anti-PD drug, rasagiline (33, 34). The findings of the Chinese Parkinson Study Group have shown a significant improvement in UPDRS scores at 6 months for patients in each dosage group, yet they were no longer significantly different at 12 months, compared with placebo patient's group (35). The authors concluded that although green tea polyphenols provide a symptomatic benefit in early untreated PD, no obvious disease-modifying effect was seen. A current clinical study with AD is recruiting patients at early stage of AD (MMSE 20-26) for a double blind 18-month clinical trial with Sunphenon, a green tea extract containing 95% EGCG, add-on to Donepezil, in the Early Stage of AD (SUN-AK study, NCT00951834 sponsored by Charite University, Berlin, Germany; completion estimated by April 2011; http://clinicaltrials.gov/ct2/show/NCT00951834). Patients will be prospectively assigned to different EGCG doses and evaluated by ADAS-COG (Score 0-70) as a primary outcome. Sunphenon has previously been shown by the authors to exert an excellent tolerability in an 18-month clinical period. However, clinical evidence demonstrating that green tea and other antioxidant polyphenols can act as protective drugs in neurodegenerative diseases is still relatively scarce, emphasizing the need of well-designed controlled studies to assess a risk reduction of PD and AD in consumers of green and black tea.

4. NEUROPROTECTIVE STUDIES WITH GREEN TEA

4.1. Preclinical *in vivo* data in aging and neurodegeneration models

Animal studies with green tea extract polyphenol fraction or its individual catechin components, have shown positive effects on cognitive and behavioral abilities during aging and in neurodegenerative conditions (9, 36). In particular, these studies have enabled to unravel the molecular mechanisms and survival pathways behind the effects of tea catechins on brain function and cognition. Research on aging has shown that 6 months administration of the antioxidant cocktail drink, β-catechin or a green tea catechin fraction, extended the mean lifespan of senescence-accelerated mouse prone-8 (SAM-P8), (37, 38) and C57BL/6 J mice (39). In old Wistar rats, green tea catechins prevented the spatial learning and memory decline after 7 months of treatment, seemingly due to the reduction of OS-related damage (40). Studies using the main green tea catechin constituent, EGCG have documented an attenuation of cognitive deficits, antioxidant

enzymes decline and apoptotic parameters in D-galactose-induced mice aging (41) and significant longevity-extending effects in Caenorhabditis elegans under stress (42). EGCG has been also shown to improve age-related cognitive decline and protect against cerebral ischemia/reperfusion injuries (43, 44), brain inflammation and neuronal damage in experimental autoimmune encephalomyelitis (EAE)(45). Recently, EGCG administration was shown to reduce the brain infarct volume of mice after transient focal ischemia accompanied by a reduction of metalloproteinase (MMP)-9 activity (46).

The positive neuroprotective effects of green tea and EGCG have been also documented in various models of neurodegenerative diseases; EGCG treatment significantly prolonged the symptom onset and life span and attenuated death signals in ALS mice model with the human G93A mutated Cu/Zn-superoxide dismutase (*SOD1*) gene (47). Similarly, a green tea polyphenol extract or individual EGCG, prevented striatal dopamine (DA) depletion and SN pars compacta (SNpc) dopaminergic neurons loss when given chronically to mice or rats treated with the parkinsonism-inducing neurotoxins, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA), respectively (48, 49). These findings receive further support from our recent animal studies, where EGCG given post-damage was shown to almost completely restore nigrostriatal DA neuron degeneration caused by MPTP (50). In terms of cognition-impaired models, long-term administration of a preparate of green tea catechins (Polyphenol E), was demonstrated to improve spatial cognition learning ability in naïve rats and prevent cognitive deficits in intracerebral amyloid beta peptide (Aβ)–damaged rats (51, 52). Additionally, EGCG reduced cognitive dysfunction in Aβ-injected mice (53) and prevented cerebral amyloidosis and impaired cognition in Alzheimer's transgenic mice (54, 55). In lipopolysaccharide-mediated neuroinflammation, oral treatment with EGCG prevented neuronal cell death and memory impairment of mice and inhibited $A\beta_{1-42}$ generation, possibly through inhibition of β- and γ-secretase (56). These findings suggest that EGCG could be a useful agent in Aβ-induced cognitive impairement and brain pathology. In a mouse model for HIV-related dementia, EGCG was found to mitigate neurotoxicity mediated by HIV-1 proteins gp120 and Tat in the presence of interferon (IFN)-gamma (57). More recently, in the 3-nitropropionic acid rat model of Huntington disease with manifest excitotoxicity and striatal degeneration, EGCG significantly attenuated behavioral alterations, oxidative damage, mitochondrial complex enzymes dysfunction and striatal damage (58).

4.2. Preclinical cell culture studies

In line with the *in vivo* findings, cell culture studies have demonstrated that flavanol catechin reduced damage 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 (59). In human neuroblastoma (NB) SH-SY5Y cells, EGCG prevented neuronal cell death caused by the neurotoxins 6-OHDA and 1-methyl-4-phenylpyridinium (MPP+) (60) and protected primary hippocampal neurons (61) and rat pheochromocytoma (PC12) cells (62) from Aβ-induced toxicity. More recently, both catechin and epicatechin were shown to protect cultured rat cortical neurons against Aβ (25-35)-induced neurotoxicity through inhibition of cytosolic calcium elevation (63). In addition, recent studies from our laboratory have demonstrated that EGCG is able to rescue and reduce mortality of NB SH-SY5Y cells when given up to 3 days after long-term serum starvation, a progressive model of apoptotic damage (64). Complementary large-scale proteomic analysis demonstrated that EGCG induced the differential expression of three major clusters of proteins related to the cytoskeleton (e.g. beta tubulin IV and tropomyosin 3), heat shock (e.g. 14-3-3 gamma, heat shock protein gp96) and metabolic energy (e.g. ATP synthase H+ transporting, mitochondrial F1 complex beta, glucosidase II beta and nerve vascular growth factor (VGF) inducible precursor) (65, 66).

5. MECHANISM OF NEUROPROTECTIVE ACTION OF GREEN TEA POLYPHENOLS

Tea catechins/flavanols are presently considered multifunctional and multitarget compounds invoking pleiotropic effects on numerous biological pathways to help keep human brain cells from dying and even help repair them. The following sections will describe salient pharmacological actions and molecular targets underlying their health benefits.

5.1. Iron chelation and antioxidant activity

There is increasing evidence that iron is involved in the mechanisms that underlie many neurodegenerative diseases (67, 68). Non-haem iron (mostly in complex with ferritin) progressively accumulates particularly in regions that are affected by AD and PD such as the putamen, motor cortex, prefrontal cortex, sensory cortex and thalamus during the first 30–35 years of life, and variable changes are observed in older individuals (67, 69). Also, during brain ageing iron is partially converted from its stable and soluble form with ferritin into haemosiderin, a partially proteolytic product of ferritin that contains iron at higher reactivity thereby increasing the risk of OS (70). Owing to its interaction with hydrogen peroxide (partly derived from excessive monoamine oxidase (MAO)-B activity in aging, AD and PD(71-73)) through Fenton chemistry, Fe^{2+}enhances generation of ROS which in turn can increase protein aggregation, including the aggregation of alpha-synuclein and Aβ, two major contributors of PD and AD pathology, respectively (Figure 3). Analysis of PD and AD brains indicates iron accumulation within specific brain regions displaying selective vulnerability to neurodegeneration, such as the substantia nigra (SN), hippocampus and cerebral cortex (74-76) in association with both intraneuronal neurofibrillary tangles (NFT), composed of hyperphosphorylated microtubule-associated protein tau (PHFτ) and Aβ-containing extraneuronal senile plaques.

The apparent link between metal dishomeostasis and neurodegenerative diseases has given rise to the conception of metal chelation therapy as a therapeutic option. In fact, a number of iron chelators/antioxidants have been shown to possess neuroprotective activity in animal models of neurodegeneration (77-82). Plant flavonoids are potent chelators of transitional metals, such as iron and copper owed to the OH at position 3 of the C ring, the OH

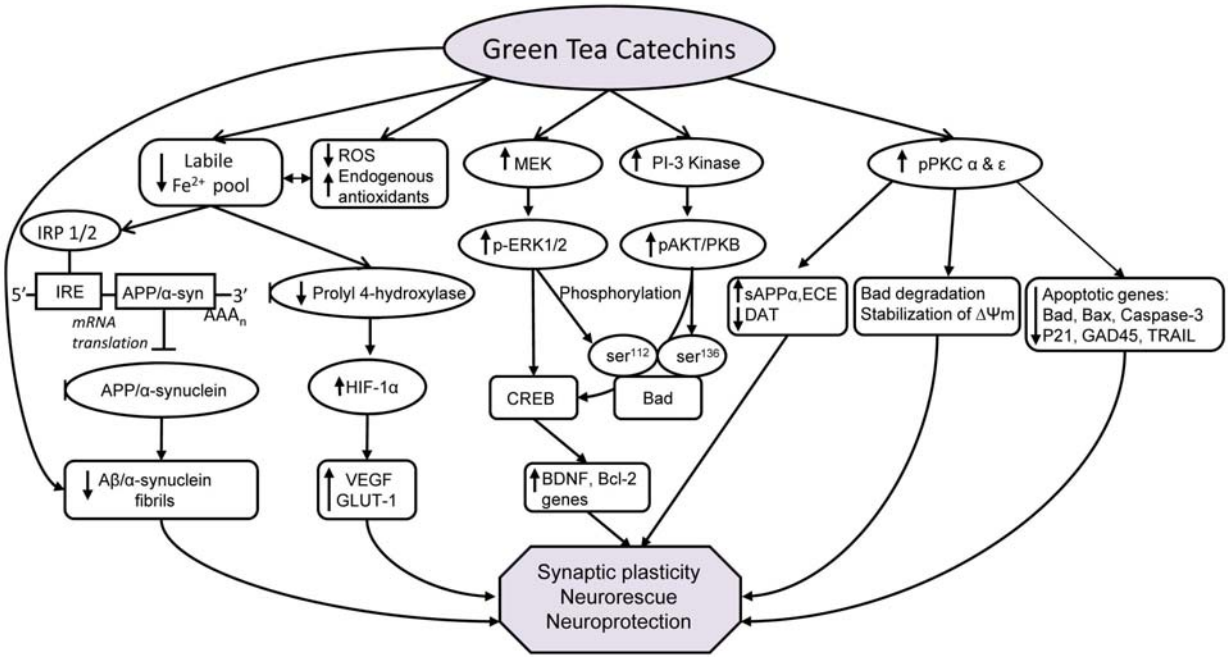

Figure 3. Schematic illustration of the proposed mechanism of neuroprotective/neurorescue action by green tea catechins. Catechins are phenolic compounds and as such, they act as powerful hydrogen-donating radical scavengers of oxygen and nitrogen species and possess the ability to complex transition divalent metal ions (Cu^{2+}, Zn^{2+}, Fe^{2+}), thereby reducing the iron pool and preventing the formation of iron-induced free radicals by Fenton chemistry. Abnormal serum iron transport to the neurons may result from a disruption in the blood-brain barrier or from release from its storage protein ferritin, thereby increasing the free-labile iron pool (ionic iron). Chelation of iron by green tea catechins can also interfere with the iron-induced degradation/inactivation of iron regulatory proteins (IRP1/2) resulting in reduced translation of amyloid precursor protein (APP) and alpha-synuclein mRNA from their 5'-UTR. Catechins may directly inhibit formation of nascent Aβ and alpha-synuclein fibrils, elongation of the fibrils, and destabilization of the formed assemblies. The net result would be a decrease in the generation and load of amyloid-beta (Aβ) peptide and alpha-synuclein fibrils. An additional target of green tea polyphenols involving iron-chelation is the inhibition of the iron and oxygen-activated prolyl-4-hydroxylases that regulate hypoxia-inducible factor (HIF)-1 stability, resulting in selective induction of cell survival genes (e.g., vascular endothelial growth factor, VEGF; glucose transporter-1, GLUT-1). The neuroprotective effect of green tea polyphenols may also involve the regulation of antioxidant protective enzymes and modulation of survival protein kinases. The latter maybe related in part to the ability of EGCG to interact with the head group region of the phospholipids within lipid bilayers and alter membrane fluidity. Indeed, EGCG induces a PKC- dependent a) fast degradation of pro-apoptotic Bad protein by the proteasome and b) prevention of mitochondrial potential collapse upon oxidative stress and reduction of apoptotic gene expression. Other PKC-accredited beneficial effects may be related to activation of α-secretase to promote generation of the non-amyloidogenic neurotrophic, soluble amyloid precursor protein-alpha (sAPPα) and activation of endothelin-converting enzyme (ECE) that degrades Aβ. Other membrane-associated signaling pathways affected by EGCG include the MEK/ERK1/2 and PI3K/AKT cascades, promoting the phosphorylative inactivation of Bad and activation of their downstream substrate, cAMP responsive element binding protein (CREB), which in turn binds to promoters of genes crucial for memory consolidation and synaptic plasticity.

at positions 3' and 4' of the B ring, or the three OH groups present in the gallol moiety of some polyphenols such as EGCG and ECG (Figure 1) (7, 83-85). In a recent study examining the differential potency of a series of polyphenols (e.g (-)-epicatechin, vanillic acid, gallic acid, quercetin, myricetin) to prevent DNA damage caused by Fe^{2+} and H_2O_2, it was found that of the 12 phenolic compounds tested, EGCG was the most potent, inhibiting over 90% of the iron-mediated DNA break (85). By correlating the pKa and IC_{50} values of phenolic compounds for inhibition of Fe_2^{+}/H_2O_2 -induced neurotoxicity, the same group (86) suggested that the binding of the polyphenols to iron was essential for their antioxidant activity.

It seems plausible that green tea polyphenols may be beneficial in aging and for treatment of PD and AD, where OS and accumulation of iron at brain areas associated with neurodegeneration have been shown (17, 18, 67, 87, 88). Tea catechins are phenolic compounds and as such, they act as powerful hydrogen-donating radical scavengers of ROS and RNS and possess the ability to chelate transition divalent metal ions (Cu^{2+}, Zn^{2+}, Fe^{2+}), thereby preventing the formation of iron-induced free radicals in *in vitro* and cell/tissue systems (7, 89). In brain tissue, green tea and black tea extracts were shown to strongly inhibit propagatory chain reaction of lipid peroxidation promoted by iron-ascorbate in homogenates of brain mitochondrial membranes (90). A similar effect was

also reported using brain synaptosomes, in which the four major polyphenol catechins of green tea were shown to inhibit iron-induced lipid peroxidation (6). In the majority of these studies, EGCG was shown to be more efficient as a radical scavenger than its counterparts ECG, EC and EGC, which might be attributed to the presence of the trihydroxyl group on the B ring and the gallate moiety esterified at the 3' position in the C ring (7).

It is suggested that *in vivo*, the neuroprotective effect of green tea polyphenols may involve the regulation of antioxidant protective enzymes. Thus, EGCG was found to elevate the activity of two major oxygen-radical species metabolizing enzymes, superoxide dismutase (SOD) and catalase in mice striatum (48). Oral administration of green tea extracts to young rats or EGCG to aged rats exhibited higher levels of antioxidant enzymes, such as glutathione peroxidase, reductase, SOD and catalase in whole brain homogenates (91, 92) and hippocampal tissue (93), compared to their respective controls. Other contribution to ROS production stems from elevated MAO-B enzyme activity. Indeed, EGCG was shown to inhibit MAO-B activity in adult rat brains (94) and C6 astrocyte cells (95). Moreover, a recent study demonstrated inhibition of nitric oxide synthase, an important ROS-generating enzyme, in the SN and striatum of MPTP mice model of PD (96). Furthermore, in peripheral tissue, it has been shown that a number of flavonoids and phenolic antioxidants at low concentrations, activate the expression of some stress-response genes, such as phase II drug metabolizing enzymes, glutathione-s-transferase and heme oxygenase-1 (HO-1), in correlation with an increase in the activity and nuclear binding of the transcription factors Nrf1 and Nrf2 to the antioxidant regulatory element (ARE) sequences contained in their promoters (97, 98).

5.1.1. Attenuation of Aβ, hyperphosphorylated τ and alpha-synuclein aggregation

The use of non-toxic, naturally occurring neuroprotective and metal-complexing flavonoids with brain access could, in theory, ''pull out iron'' from those brain areas where it preferentially accumulates and alleviate the brain from free-reactive iron overload by directly influencing aggregation and deposition of Aβ and alpha-synuclein in brains of AD and PD patients, respectively. Iron was demonstrated to facilitate the aggregation of Aβ and induce aggregation of the major constituent of NFTs, hyperphosphorylated τ (Tau) (99, 100). *In vitro* studies demonstrated that Aβ has high affinity for iron, and the iron-binding sites are located in the hydrophilic N-terminal part of the peptide (101). When neuronal cells were exposed to Aβ in the presence of the iron chelator desferroxamine, toxicity was significantly attenuated, while conversely, the toxicity was restored to original levels following incubation of Aβ with excess free iron (102), suggesting a redox-active iron-mediated effect. Similarly to Aβ, alpha-synuclein has been shown to form 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(103, 104).

In addition to the attenuation of iron- and OS-mediated peptide aggregation, natural flavonoids were shown to bind directly to Aβ and alpha-synuclein nascent fibrils or mature aggregates and impair their stability. Indeed, wine polyphenols (e.g., resveratrol, myricetin, quercetin, kaemferol), curcumin, (+)-catechin, (-)-epicatechin, nordihydroguaiaretic acid, and rosmarinic acid, were shown to inhibit formation of nascent Aβ and alpha-synuclein fibrils, elongation of the fibrils, and destabilization of the formed assemblies (105, 106). EGCG was reported to interfere with an early step in the amyloid formation cascade by binding directly to the natively unfolded alpha-synuclein and Aβ polypeptides, thus inhibiting their fibrillogenesis and redirecting them into an alternative "off pathway" before they become toxic (Figure 3) (107). A similar effect was observed with resveratrol (108). In effect, partial aggregated and oligomerized intracellular Aβ was documented to be cytotoxic and synaptotoxic in cell culture and impair memory in animal studies (109). Similarly, a truncated form of alpha-synuclein showed increased aggregation into large inclusions bodies, increased accumulation of high molecular weight alpha-synuclein species, and enhanced neurotoxicity in transgenic Drosophila (110).

In addition to its early effect on Aβ and alpha-synuclein fibrillogenesis, EGCG was lately shown to convert large, mature alpha-synuclein and Aβ fibrils into smaller, amorphous non-toxic protein aggregates and this effect was evident only with catechins carrying a gallate moiety such as ECG and EGCG (111), the group in the molecule suggested to complex iron. Thus, it is possible that the direct binding of iron-complexing flavonoids to Aβ via the gallate group, plays a major role in the disruption and solubilization of the plaques in AD animal model and *in vitro* aggregates. In this regard, another study showed that only gallate forms of catechins were able to protect hippocampal cells against amyloid-induced toxicity (112).

5.1.2. Inhibition of amyloid precursor protein (APP) translation

Another outcome of transition metal-complexing molecules, including EGCG, is related to their ability to reduce Aβ formation via translational suppression of its generator, amyloid precursor protein (APP), a type I integral membrane protein. This effect is associated with modulation of an iron responsive element (IRE) located at APP 5'UTR-mRNA (113, 114). In mouse hippocampus, EGCG was shown to down-regulate membrane-associated APP protein level (115). In SH-SY5Y cells EGCG reduced full-length APP, without altering APP mRNA levels, while exogenous iron supplementation reversed its effect (113). These results suggest a post-transcriptional action, presumably by the mechanism of chelating intracellular iron pools and APP mRNA post-transcriptional inhibition (Figure 3). This is further supported by the observation that EGCG suppressed translation of a luciferase reporter gene driven by the IRE-type II-containing sequences of APP (113). Furthermore, it was found that EGCG markedly reduced secreted Aβ levels in the conditioned medium of Chinese hamster ovarian cells, overexpressing "Swedish" mutated APP (CHO/ΔNL) (113) and in primary neuronal cells derived from transgenic mice bearing the APP "Swedish" mutation (54). Recently, Friedlich *et al* (116)

have described a putative IRE in the 5'-UTR of PD-related alpha-synuclein mRNA and predicted that this RNA structure may have the potential to function as a post-transcriptional regulator of α alpha-synuclein protein synthesis in response to iron and redox events, in a pattern that resembles that of APP and the iron-associated protein ferritin (67, 114).

5.1.3. Modulation of iron-dependent hypoxia-inducible factor-1

An additional level of neuroprotection by iron-complexing agents involves the stabilization of the transcriptional activator, hypoxia-inducible factor (HIF)-1 and expression of its target protective genes, such as erythropoietin, vascular endothelial growth factor (VEGF), enolase, transferrin receptor and heme oxygenase. HIF-1 is a heterodimeric transcription factor, composed of two subunits: HIF-1α, an oxygen-labile protein that becomes stabilized under hypoxic conditions and HIF-1β, which is constantly expressed (117). HIF is highly involved in the pathology of a number of diseases associated with tissue hypoxia (reduced oxygen tension) (118). Within the cells, HIF-1 is under the control of a class of iron-dependent and oxygen-sensor enzymes, HIF prolyl-4-hydroxylases (PHDs) that target the regulatory alpha subunit of HIF-1 for degradation by the proteasome (117) (Figure 3). The possibility of modulating HIF-1 activity by targeting the free-labile intracellular iron pool by iron chelators and inhibitors of PHDs is gaining recognition, posing HIF-1 as a potential therapeutic target for neurodegenerative diseases (119-121). Similar to hypoxia, natural flavonoids were demonstrated to induce intracellular accumulation of HIF-1α through activation of enzyme systems and signaling pathways, such as phosphatidylinositide 3'-OH kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK, e.g. extracellular signal-regulated protein kinase, (ERK)), as well as by a direct complexing of iron and inactivation of PHDs (84, 122-124). For example, the main constituent of the standardized dietary flavone, Ginkgo biloba (Ginkgoaceae) extract, EGb 761, was demonstrated to protect hypoxic PC12 cells and up-regulate HIF-1 α protein expression through the activation of the p42/p44 MAPK pathway(125). Studies with different cell lines demonstrated that a number of flavonoids including luteolin, fisetin and quercetin, induced HIF-1α levels under normal oxygen pressure and this effect was structurally related to the capability of the molecules to efficiently bind intracellular iron (124, 126, 127). Also, EGCG and ECG were shown to induce HIF-α protein and HIF-1 activity and increase the mRNA expression levels of its pro-survival gene targets glucose transporter-1 (GLUT-1), VEGF, and $p21^{waf1/cip1}$, whereas this effect was blocked by iron and ascorbate, indicating that these catechins may activate HIF-1 through the chelation of iron (128, 129). Similar results were reported for the flavonoid quercetin(84).

Another link between hypoxia and iron is reflected by the hypoxic-mediated positive regulation of two ubiquitously expressed, homologous members of the aconitase gene family, the iron regulatory proteins (IRPs), IRP1 and IRP2 (130) and consequential transactivation or inhibition of their target mRNAs, such as AβPP, transferrin receptor, ferritin and ferroportin. The mRNA binding activity of IRP2 is regulated at the level of protein abundance, through iron dependent degradation by the ubiquitin-proteasome pathway. This degradation is also facilitated by oxygen. Thus, at low cellular iron and oxygen levels, IRP2 is stabilized and able to bind IREs on target mRNAs (131). Interestingly, the free iron-induced proteasomal-mediated degradation of IRP2 involves also activation of a PHD and is inhibited by iron chelators (132, 133). Thus, it is possible that IRP2 and HIF-1 protein abundance is modulated by a common pathway. The reduction in the chelatable iron pool by EGCG may result in the inhibition of PHD activity and consequently, in the concerted activation of both HIF and IRP2.

5.2. Modulation of cell signaling pathways by green tea polyphenols

Originally viewed as simple antioxidants/anti-inflammatory, plant-derived flavonoids including the main flavanol polyphenol of green tea, EGCG are currently suggested to selectively activate kinase signaling molecular pathways implicated in neuronal plasticity, regeneration and survival. It cannot be ruled out, however, that some of these effects may have resulted from interplay between their scavenging/neutralization action of ROS/NOS and consequential attenuation/induction of related signal transduction pathways.

5.2.1. Activation of the protein kinase C (PKC) pathway

Our previous *in vitro* cell signaling studies revealed a specific involvement of the PKC pathway in the neuroprotective mechanistic action of EGCG (60, 134). PKC has a fundamental role in the regulation of cell survival, programmed cell death, long-term potentiation (LTP) (135) and consolidation of different types of memory (136, 137). Additionally, the induction of PKC activity in neurons has been shown to protect against various exogenous insults, such as oxygen/glucose deprivation in organotypic slice cultures (138) or Aβ toxicity in rat cortical or hippocampal neurons (139, 140).

A more direct demonstration of a possible interaction between green tea catechins and PKC has been provided by the study of Kumazawa *et al* (141) employing solid-state nuclear magnetic resonance and showing that EGCG interacts with the head group region of the phospholipids within lipid bilayers from liposomes and moves freely on the membrane surface (142). The interaction pattern of EGCG in terms of rotational motion within the lipid bilayers was similar to that described for the phorbol ester 12- *O* -tetradecanoylphorbol-13-acetate (TPA) (143), a prototype activator of PKC. This suggests that a direct interaction of green tea catechins with cell membranes may result in the rapid PKC activation shown previously by our group (60) (Figure 3). Furthermore, the impact of EGCG on membrane fluidity may give rise to activation of other membrane-associated signaling pathways.

The distinct activation of PKC isoforms/pathway has been recently reported also for curcumin, the natural phytochemical obtained from dried

root of Turmeric (Curcuma Longa), which was shown to compete with Ca^{2+} for the regulatory domain of PKC (144). Fluorescence spectroscopy and molecular modeling studies found that curcumin binds to the N-terminal region of PKCδ, PKCε and PKCθ with an EC_{50} and fluorescence emission similar to that of TPA (145).

The following sub-sections will present topical literature on the involvement of PKC signaling system in the modulation of apoptotic molecular mediators, mitochondria integrity, APP processing and DA synaptic function by polyphenols.

5.2.1.1. Prevention of apoptosis and mitochondrial dysfunction

The antiapoptotic/neuroprotective actions of green tea catechins seem to be attained at low micromolar concentrations, whereas high doses have been shown to exert anti-proliferative, anti-angiogenic and pro-apoptotic actions, thus having implications in cancer management (60, 146). A rapid phosphorylative activation of PKC by low micromolar concentrations of EGCG is thought to be the main mechanism accounting for its neuroprotective activity against several neurotoxins, such as Aβ (147), serum withdrawal (62, 64), and 6-OHDA (60) and for its neurorescue effect against long-term growth factors withdrawal (64). The neurorestorative effect was demonstrated to involve reduction of the apoptotic markers, cleaved caspase 3; its downstream cleaved substrate poly-ADP-ribose-polymerase (PARP), a nuclear zinc finger DNA-binding protein that detects and binds to DNA strand breaks; Bad, a member of a group of "BH3 domain only" proteins of the Bcl-2 family (64). This was accompanied by a rapid translocation of the isoform PKCα (particularly important in neuronal growth and differentiation in the brain), to the neuronal membrane compartment (64, 148). Accordingly, upon pharmacological inhibition of PKC activity, EGCG could not overcome the neuronal death, suggesting that this cascade is essential for the neuroprotective and neurorescue effects of EGCG. *In vivo*, it was also shown that a 2 weeks treatment of *naïve* with EGCG prevented the extensive depletion of PKCα and counteracted the robust increase of pro-apoptotic Bax protein in the striatum and SN of mice intoxicated with MPTP (149). Similarly, a particular involvement of PKC activity was shown for the wine polyphenol, resveratrol against Aß toxicity in hippocampal neurons, while PI3K or MAP kinases signaling pathways were not activated by the polyphenol (140).

Recently, we have identified a novel PKC-linked pathway in the neuroprotective mechanism of action of EGCG, which involves a rapid PKC-mediated degradation of Bad protein by the UPS in NB SH-SY5Y cells (134). Bad has been suggested to link survival signals to the mitochondrial cell death machinery. It may directly contribute to the opening of the mitochondrial megachannel permeability transition pore by its heterodimerization with the mitochondrial death suppressor proteins Bcl-2 and/or Bcl-xL, thus neutralizing their antiapoptotic function (150). In this respect, one month administration of EGCG (2 mg/kg) to aged (> 24 months) male Wistar rats augmented the activities of Kreb's cycle enzymes and electron transport chain complexes in brain mitochondria, decreased the expression of hydroxynonenal in aged brain, and up-regulated the antioxidant system (151). These results support an antioxidant potential of EGCG at the level of mitochondria. Indeed, a recent study suggests that EGCG could directly scavenge mitochondrial generated free radicals, as it was found to accumulate in mitochondria and selectively protect rat cerebellar granule neurons from apoptosis induced by various generators of OS (152). This may partly account for the attenuation of depolarization of the inner mitochondrial membrane potential by a polyphenol-rich green tea extract or EGCG, following oxygen-glucose deprivation in C6 glial culture (153).

5.2.1.2. Regulation of APP and DA metabolism

APP can be processed via alternative pathways: a non-amyloidogenic secretory pathway that includes cleavage of APP to sAPPα by a putative α-secretase within the sequence of the amyloidogenic Aβ peptide, thus precluding the formation of Aβ; the second pathway generates Aβ via the sequential action of β- and γ-secretases (154). Previous studies have demonstrated that either short- or long-term incubation with EGCG promotes the generation of the non-amyloigogenic, soluble form of APP, sAPPα, via PKC-dependent activation of α-secretase (113, 147). In contrast to Aβ, sAPPα possesses neuroprotective activities (155) and promotes neurite outgrowth (156) and synaptogenesis (157). Since sAPPα and Aβ are formed by two mutually exclusive mechanisms, it can be assumed that stimulation of the secretory processing of sAPPα might prevent the formation of the amyloidogenic Aβ (Figure 3). New supportive data emerged from a study conducted in an Alzheimer's transgenic mice model, showing that EGCG promotes sAPPα generation through activation of α-secretase cleavage (54) (158). This was accompanied by a significant reduction in cerebral Aβ levels and β-amyloid plaques. These results were supported *in vitro* by the observation that exogenously added EGCG to primary neurons derived from the above transgenic mice bearing the APP "Swedish" mutation, markedly reduced secreted Aβ levels in the conditioned medium (54). More recently this research group reported that encapsulation of EGCG into nanolipidic particles markedly improved its neuronal α-secretase enhancing ability *in vitro* and its oral bioavailability *in vivo* over free EGCG (159).

Other potential beneficial effect of PKC activation in AD is related to the recent finding showing that neuronal overexpression of PKCε in transgenic mice expressing familial AD-mutant forms of the human APP, decreases Aβ levels and plaque burden and this is accompanied by increased activity of endothelin-converting enzyme (ECE), which degrades Aβ (160). Thus, it can be hypothesized that since EGCG has been shown to increase the levels of PKC isoforms α and ε in mice hippocampus and striatum (147, 149), EGCG may reduce Aβ levels in AD pathology, both via concomitant stimulation of sAPPα secretion and promotion of Aβ clearance through increased ECE activity (Figure 3). Despite the general support for the

amyloid hypothesis as a key contributor to neuronal death and dementia in AD, a direct connection between Aβ deposits in senile plaques and neurodegeneration has not been yet established. In this respect, Alzheimer's transgenic mice with Aβ deposits do not exhibit significant neuronal loss in hippocampus and association cortex (161). Therefore, the ultimate proof for the effectiveness of drugs, capable of lowering or eliminating brain Aβ aggregation/accumulation will be their clinical benefit.

PKC-dependent pathway activation was also implicated in the regulatory effect of EGCG on DA presynaptic transporters (DAT) internalization (162). EGCG caused a dose-dependent inhibition of DA uptake in DAT-PC12 and decreased membrane-bound DAT, while this effect was abolished by blockade of the PKC pathway This observation, together with the finding that EGCG inhibited catechol-O-methyltransferase (COMT) activity in rat liver cytosol homogenates (163), may be of particular significance for PD patients given that DA and related catecholamines are physiological substrates of COMT.

5.2.2. Modulation of other Signal Transduction Pathways

In addition to PKC, other cell signaling pathways have been implicated in the action of green tea catechins, such as mitogen activated protein kinase (MAPK, e.g. ERK1 and 2) and PI3K/AKT signaling cascades (36, 115), known to be connected to attenuation of neuronal death and cellular injury by OS (164-166) (Figure 3). Previous *in vitro* studies demonstrated the potency of EGCG to induce antioxidant responsive element (ARE)-carrying stress genes, such as phase II drug metabolizing enzymes, glutathione-s-transferase and heme-oxygenase1, likely via activation of the MAPK pathway cell survival signaling regulators, as p44/42 ERK 1/2, JNK and p38 MAPK (97, 167). Thus, green tea catechins may activate MAPK signaling cascades to normalize ERK activity caused by exogenous OS-inducing agents. Indeed, it was shown that EGCG counteracted the decline in ERK1/2 induced by 6-OHDA in NB SH-SY5Y cells (60) and induced phosphorylation of ERK1/2 in serum-deprived SH-SY5Y cells (65). In rat cortical neurons, the flavanol (-)epicatechin induced a concentration-dependent phosphorylation of PI3-K, ERK1/2 and their downstream substrate, cAMP responsive element binding protein (CREB) and subsequently increased CREB-regulated gene expression (146). CREB is a transcription factor that binds to the promoter regions of many genes associated with memory and synaptic plasticity, playing a crucial role in long-term potentiation (LTP) and long-term memory formation (168, 169). In agreement with the *in vitro* findings, chronic treatment of two different strains of aged mice with green tea catechins promoted improvement in learning tasks and memory deficits, associated with increased levels of CREB phosphorylation in the hippocampus and increased expressions of brain-derived neurotrophic factor (BDNF) and Bcl-2, two target genes of CREB, having regulatory roles in synaptic plasticity (38, 39). Similar gene alterations were recently reported in hippocampus of old rats after prolonged administrations of green tea infusion (93). Also, 5 weeks oral administration of l-Theanine, an amino acid component of green tea to mice, was reported to attenuate cognitive dysfunction and neurotoxicity induced by intracerebroventricular injection of $A\beta_{1-42}$ via inhibition of MAPK/p38 and the inflammatory nuclear factor κB (NF-κB) pathways (170).

6. COMBINATION THERAPY WITH GREEN TEA POLYPHENOLS TARGETING DISTINCT BRAIN PATHOLOGIES

The poly-etiological origin of AD and PD, suggests that the ability to modulate several targets at once could be beneficial for these diseases. This has laid the basis for the assumption that a single drug possessing two or more active neuroprotective moieties, or a combination of compounds, targeted at multiple pathological aspects of the same disease, may offer a superior therapeutic benefit. In line with this rationale, current therapeutic strategies in neurodegenerative diseases entail combination of available drugs in the market ((e.g. acetyl cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) antagonist in AD; levodopa and dopaminergic agonist in PD)) and addition of nutritional supplements to the diet such as antioxidant vitamins, CoQ_{10}, fish oil, EGCG-enriched capsules, folate, vitamin B6, alpha-tocopherol, S-adenosyl methionine, N-acetyl cysteine, acetyl-L-carnitine and ginko biloba extracts together with changes in lifestyle and behavior (171-177).

Following this therapeutic strategy rationale, we have recently selected the anti-PD drug, rasagiline (Azilect®), a second generation, irreversible selective inhibitor of MAO-B and EGCG for an *in vivo* preclinical neurorescue/neurorestorative drug cocktail study. Mounting evidence from preclinical, epidemiologic and human clinical trials, indicates that both compounds exert a neuroprotective action (26, 29-32, 34, 48, 178-182). The underlying principle was that the distinct neuroprotective/neurorescue pharmacological profile of rasagiline would complement that of EGCG and restore the neuronal loss and molecular targets damaged in animal parkinsonism. Our results revealed that subliminal doses of rasagiline and EGCG, which individually have no profound protective effect, synergistically restored DA neurons in the SNpc and replenished SNpc serine/threonine kinase Akt/PKB levels and striatal DA, when given to mice after MPTP lesion(50). In analogy to PD, a recent study conducted in Tg2576 mice model of AD showed that co-treatment with the nutritional supplements fish oil (8mg/kg/day) and EGCG (12.5mg/kg/day) synergistically inhibited cerebral Aβ deposits (183). Similarly, in the triple transgenic mice model of AD (βAPPSwe, presenilin-1M146V and tauP301L) dietary treatment with fish oil/omega-3 fatty acids, curcumin, or a combination of both has the potential to improve insulin/trophic factor pathway defects, (increased phospho-insulin receptor substrate-1 IRS-1 and decreased total IRS-1) and cognitive deficits that are observed in AD (184). Also, a nutraceutical combination of polyphenols from blueberry and green tea, the amino acid carnosine and vitamin D3, has been shown to improve neural stem cell proliferation in the subventricular zone in aged rats and cognitive function in aged rats (185). In a mouse excitotoxic injury model a

combination of tea polyphenol and memantine (an NMDA receptor antagonist), but not each compound alone, improved the impaired locomotor activity (50, 182).

This further support the view that moderate diet supplementation with nutritional additives could be of significant therapeutic value for the treatment of aging, neurodegenerative diseases and clinical excitotoxic injury such as brain trauma, brain ischemia and epilepsy.

7. PERSPECTIVE

The comprehension of the health beneficial effects of diets rich in fruits and vegetables could contribute to a healthier aging process and decreased prevalence of cognitive deficits and common neurodegenerative diseases. Possibly, the daily intake of dietary plant flavonoids will constitute a prophylactic treatment long before the onset of symptoms in people at risk of getting AD, PD or other neurological diseases with neuronal degeneration (family history, genetic predisposition) and to delay the cognitive decline and other deterioration processes in aging.

It becomes evident that neurodegenerative disorders will require multiple target therapy to address the varied pathological aspects of the disease. Thus, it is not surprising that a large amount of drugs/agents in therapeutic use for these conditions are antioxidants/metal complexing, bioenergetic and anti-inflammatory agents. Therefore, the multi-pharmacological activities of green tea catechins, added to their non-toxic, brain permeable lipophilic nature may be of significance for neuroprotection/neurorescue. Presently, their suggested potential cellular sites of action include a direct neutralization of free-radicals induced OS, attenuation of reactive free-iron pool and consequent reduction in APP/alpha-synuclein translation, modulation of the activity of endogenous antioxidant detoxifying enzymes, pro-survival protein kinases, metal and calcium homeostasis, transcription factor(s) activation, mitochondrial integrity and promotion of neurite outgrowth. It is plausible that some of the actions result from a common effect of green tea polyphenols on one or more of the kinases and/or their upstream activators.

An additional novel approach entails drug combination or poly-pharmacology, providing a practical way to design specific neuroprotective therapy. The complex symptomatology of neurodegenerative diseases often necessitates the use of more than one drug (monotherapy). New emerging preclinical evidence suggest that in a combination therapy regime, green tea EGCG may have the potential to complement the pharmacological activities of current drugs in use for neurodegenerative diseases, such as rasagiline and memantine (50, 182). These findings underline the importance of having a clear understanding of green tea catechins molecular mechanism of action, in the evaluation of its potential in a polypharmacy paradigm, with drugs possessing distinct neuroprotective pharmacological profile, in the quest for a disease modifying therapy.

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