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ORIGINAL ARTICLE

Neuroprotective and Anti-inflammatory Properties of a Coffee Component in the MPTP Model of Parkinson's Disease

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Abstract Consumption of coffee is associated with reduced risk of Parkinson's disease (PD), an effect that has largely been attributed to caffeine. However, coffee contains numerous components that may also be neuroprotective. One of these compounds is eicosanoyl-5-hydroxytryptamide (EHT), which ameliorates the phenotype of α-synuclein transgenic mice associated with decreased protein aggregation and phosphorylation, improved neuronal integrity and reduced neuroinflammation. Here, we sought to investigate if EHT has an effect in the MPTP model of PD. Mice fed a diet containing EHT for four weeks exhibited dose-dependent preservation of nigral dopaminergic neurons following MPTP challenge compared to animals given control feed. Reductions in striatal dopamine and tyrosine hydroxylase content were also less pronounced with EHT treatment. The neuroinflammatory response to MPTP was markedly attenuated, and indices of oxidative stress and JNK activation were significantly prevented with EHT. In cultured primary microglia and astrocytes, EHT had a direct anti-inflammatory effect demonstrated by repression of lipopolysaccharide-induced NFκB activation, iNOS induction, and nitric oxide production. EHT also exhibited a robust antioxidant activity in vitro. Additionally, in SH-SY5Y cells, MPP^+-induced demethylation of phosphoprotein phosphatase 2A (PP2A), the master regulator of the cellular phosphoregulatory network, and cytotoxicity were ameliorated by EHT. These findings indicate that the neuroprotective effect of EHT against MPTP is through several mechanisms including its anti-inflammatory and antioxidant activities as well as its ability to modulate the methylation and hence activity of PP2A. Our data, therefore, reveal a strong beneficial effect of a novel component of coffee in multiple endpoints relevant to PD.

Kang-Woo Lee and Joo-Young Im contributed equally to this work.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder associated with significant disability and impaired quality of life. Loss of dopaminergic neurons in the substantia nigra (SN) and depletion of the transmitter dopamine in the striatum are responsible for the cardinal motor manifestations of bradykinesia, tremor and rigidity. A consistent indicator of neuronal injury found in postmortem studies is evidence for oxidative and nitrative stress with damage to proteins, lipids, and DNA [1]. A major source of reactive oxygen species (ROS) and reactive nitrogen species in the SN are neuroinflammatory cells that are a well-established pathologic feature of PD [2, 3]. These reactive microglia are believed to play a critical role in the progressive neuronal degeneration. High levels of various reactive species damage cellular organelles including mitochondria, particularly inhibiting complex I of the respiratory chain, resulting in energy depletion, release of cytotoxic mediators and neuronal death. Impaired complex I activity, in part due to the oxidation of its catalytic subunits, occurs in the SN and other tissues in PD [4]. To model this pathobiology, the complex I inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent toxin that damages dopaminergic neurons and produces a parkinsonian phenotype in mice and primates [5]. Therapeutics that target these pathogenetic events, including inflammation and oxidative stress, can potentially alter the rate of neurodegeneration in PD.

We recently reported that eicosanoyl-5-hydroxytryptamide (EHT) is protective in a model driven by α-synuclein, a key protein in PD [6]. In the brains of α-synuclein transgenic mice, EHT ameliorates α-synuclein aggregation and phosphorylation, improves neuronal function and markedly reduces the associated neuroinflammation following chronic oral administration. These indices of a favorable neuropathological response to EHT are accompanied by improved behavioral performance. EHT functions, at least in part, by promoting the methylation of phosphoprotein phosphatase 2A (PP2A) by directly inhibiting the interaction between methylated PP2A and its specific methylesterase PME-1 [6].

Interestingly, EHT is a component of coffee, which has been the subject of numerous epidemiological studies to assess its association with modified risk of developing an array of conditions including neurodegenerative disorders such as PD [7–11]. Prior studies about the beneficial effects of coffee in PD have focused largely on caffeine [8, 10]. But coffee contains over 1,000 compounds, and the central bioactivity of EHT in an α-synuclein transgenic mouse model of PD [6] suggests that constituents of coffee other than caffeine may have neuroprotective effects. In further support of this notion is the demonstration that decaffeinated coffee is protective in Drosophila models of PD [12].

In the present investigation we sought to determine if the demonstrated efficacy of EHT in an α-synuclein-based model of PD extends to the toxic MPTP model. We show that EHT indeed protects against the toxicity of MPTP and does so through multiple actions including as an anti-inflammatory agent, anti-oxidant and PP2A modulator, which are all relevant mechanisms to neurodegenerative diseases.

Methods

Antibodies

Antibodies to the following markers and their sources were: tyrosine hydroxylase (TH) (1:2,000) and β-actin (1:10,000) from Sigma-Aldrich (St. Louis, MO); GFAP (1:3,000) from DAKO (Glostrup, Denmark); Iba-1 (1:2,000) from WAKO (Richmond, VA); phospho-JNK (1:1,000), phospho-p65 (1:1,000) and p65 (1:1,000) from Cell Signaling (Beverly, MA); total JNK (1:1,000) from Santa Cruz Biotechnology (Santa Cruz, CA); iNOS (1:500) from BD Biosciences (San Diego, CA); unmethylated-PP2A 4b7 (1:2,000) and total PP2A C subunit (Catalog # 06–222) (1:2,000), both from Millipore (Temecula, CA).

Animals and Treatment

Male C57BL/6 mice were housed two per cage under a 12-hour dark–light cycle with ad libitum access to food and water. Before MPTP treatment, 8-week old mice were placed on a diet containing 0.01 % EHT (n=24), 0.1 % EHT (n=24) or control chow (n=24) for 4 weeks. With *ad libitum* access to feed, these EHT concentrations translate to 12 mg/kg/day and 120 mg/kg/day, respectively. The animals were then challenged with four injections of 10 mg/kg MPTP (or saline) intraperitoneally at 2-hr intervals on one day and sacrificed 3 days or 7 days after the last injection. MPTP- and saline-injected mice were anesthetized with avertin and perfused transcardially with sterile saline. Brains were quickly harvested, and one hemibrain was immediately frozen in liquid nitrogen for biochemical studies and the other hemibrain post-fixed in 4 % paraformaldehyde for immunohistochemical stains. For TH immunohistochemistry and stereology, anesthetized mice were perfused with sterile saline followed with freshly prepared 4 % paraformaldehyde. Brains were removed and post-fixed in the same fixative at 4 °C overnight. All animal procedures were

approved by the UMDNJ – Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Stereological Nigral Dopamine Neuron Counts

Unbiased counting of TH-positive dopaminergic neurons within the substantia nigra (SN) was performed as described previously [13]. Briefly, coronal 40-μm-thick sections were cut through the SN on a vibratome. Every fourth free-floating section was stained with an antibody against TH and processed with the ABC method (Vector Laboratories, Burlingame, CA). TH-stained sections were counterstained with Nissl and mounted with the appropriate mounting medium. The numbers of TH immunoreactive cells and Nissl-positive cells in the SN pars compacta (SNpc) of one hemisphere were counted using an optical fractionator method. This stereological method of cell counting is not affected by either the reference volume (SNpc) or the size of the counted elements [14]. For each mouse brain, five selected representative sections of the SNpc were analyzed in a STEREOLOGER – Computer-Assisted Stereology System (Applied Scientific Investigation) consisting of a Leica upright microscope (Leica DM 2000) equipped with a computer-controlled ASI MS-three axis stage system, a Baumer FireWire Digital Color Camera, and the Stereologer Software (2.0) program. These analyses were done by an individual completely blind to the treatment group of each brain.

HPLC and ELISA

Striatal dopamine and homovanillic acid (HVA) levels were measured by HPLC with electrochemical detection as described previously [15]. MPP^+ levels were measured by HPLC using UV detection. Striatal TH content was measured by an enzyme-linked immunosorbent assay as described before [16].

Western Blotting and Immunohistochemistry

Western blot analysis and immunostaining were performed as previously described [6]. For Western blots, mouse brain tissue was homogenized in 4 °C lysis RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate) containing phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA) and protease inhibitor cocktail set V (Calbiochem, La Jolla, CA), centrifuged at 14,000 rpm at 4 °C for 30 min, and the supernatant recovered. Separated proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA), which was then blocked with 5 % non-fat dry milk in Tris-buffered saline and 0.1 % Tween 20. Immunoblots were detected using ECL plus (PerkinElmer, Boston, MA). For immunohistochemistry, fixed brains were cut coronally into 40-μm-thick sections using a vibratome. Free-floating sections were blocked by 5 % BSA. Biotinylated HRP complex (Vector Laboratories, Burlingame, CA) and 3.3'-diaminobenzidine were used for color development. Images of stained sections were captured using a Nikon Eclipse 55i microscope and NIS Elements D3.2 software (Nikon), and analyzed using NIH ImageJ (versions 1.45 s). Analysis of the in vivo data was confirmed by blinded members of the research team.

Measurement of Reduced Glutathione and Oxidized Glutathione

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in the substantia nigra and cerebral cortex by the GSH assay kit (Cayman) using enzymatic recycling. Briefly, tissues were suspended in 2-(N-morpholino)ethanesulphonic acid (MES) buffer and homogenized in a Teflon-glass Potter homogenizer. Homogenates were centrifuged at 10,000x *g* for 15 min. Subsequently, the supernatant was mixed with equal volume of metaphosphoric acid (MPA, Sigma-Aldrich) and 50 ul of triethanolamine (TEAM, Sigma-Aldrich) was added per ml of sample. For measurement of GSSG, 10 μl of 2-vinylpyridine solution was added to each sample or standard, and then 50 μl was mixed with 150 μl of assay Cocktail (MES buffer, DTNB, NADPH, and glutathione reductase). Absorbance was measured at 405 nm. GSH concentration of each sample was calculated as nmol/mg protein. BCA method was used to measure protein content.

Antioxidant Activity Assay

The total antioxidant capacity of EHT was estimated in vitro using the colorimetric antioxidant assay kit (ABTS method) obtained from Cayman Chemical Company (Ann Arbor, MI). ABTS (2, 2'-Azino-bis-[3-ehthylbenzthiazoline sulphonate]) was used as the chromogen, which changes into a colored monocation radical form ($ABTS^{\bullet+}$) by the oxidative agent metmyoglobin, and monitored by measuring absorption at 750 nm. Addition of antioxidants to the mixture reduces $ABTS^{\bullet+}$ into a colorless form as a function of concentration. The relative antioxidant activity of EHT was compared with those of ascorbic acid (Sigma Co., St. Louis, MO) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E. Compound solutions were prepared in ethanol or water and subsequently diluted in assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9 % sodium chloride and 0.1 % glucose) for introduction into the assay. ABTS inhibition dose–response curves were generated by fitting data using SigmaPlot for each of three independent experiments, and EC_{50} values were determined from these plots.

Primary Microglia and Astrocyte Cultures

Primary microglia and astrocytes were cultured as described previously [17, 18] with some modifications. Briefly, CD-1 (ICR) neonatal mice (day 0–1; timed-pregnant females purchased from Charles River Laboratories) were sacrificed and cortices were isolated. Dissociated cortical cells were plated in minimal essential medium (MEM) supplemented with 20 mM glucose, 5 % fetal bovine serum, 5 % horse serum, 2 mM glutamine, 100 ug/ml streptomycin, and 100 U/ml penicillin at a density of 2×10^5/ml onto poly-D-lysine (20 ug/ml)-coated T75 plates. After 10–14 days in culture, astrocytes and microglia were isolated. Microglial cells were separated by shaking the culture plate for 30 min at 200 rpm, plated in 24-well plates ($1X10^5$ cells per well), and allowed to settle for 1 h. Non-adherent cells were removed by washing with the same medium. Adherent cells were used for experiments 24 h after plating. Astrocytes were separated by shaking the culture plate for 18 h at 200 rpm and then plated in 24-well plates ($1X10^5$ cells per well) and allowed to settle for 10 days.

Measurement of Nitric Oxide Levels

Primary cultures were pre-treated with EHT for 1 h and then challenged with 1 μg/ml bacterial lipopolysaccharide (LPS) (serotype 0111:B4) (Sigma-Aldrich). Nitric oxide production was assessed by measuring nitrite levels in the medium using the Griess Reagent System (Promega) according to the manufacture's protocol. A Nitrite Standard reference curve was prepared for each assay for accurate quantification in the experimental samples.

Cell Culture and Cell Death Assay

Human neuroblastoma cell line SH-SY5Y was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS). Cells were pretreated with 10 or 25 μM of EHT or vehicle for 6 h and then incubated with MPP^+ for 18 h. Cell death was assessed by measuring LDH release from damaged cells using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. For Western blot analyses, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed with 1 % SDS containing protease and phosphatase inhibitors (Roche, Basel, Swiss). Lysates were sonicated three times for 10 s each and their protein contents were determined using a BCA assay kit.

Statistical Analysis

Data are presented as means ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple range test. Significance was determined at $p<0.05$.

Results

EHT Protects Mice Against MPTP Toxicity

Pre-treating mice with EHT for 4 weeks prior to challenging them with MPTP showed robust protection of nigral dopaminergic neurons in a dose-dependent manner. Unbiased stereology of nigral TH positive neurons performed seven days post-MPTP showed significant neuroprotection by EHT treatment. Compared with 66 % decline in the number of TH positive neurons following MPTP in control diet-fed mice, this measure declined by only 46 % in low dose EHT (0.01 % of diet) treated animals and by 27 % in high dose EHT (0.1 % of diet) treated animals (ANOVA $p<0.05$) (Fig. 1a and c). Similarly, Nissl stained neurons were depleted significantly by MPTP lesioning but not in animals that had been pretreated with high dose EHT (Fig. 1b). The numbers of both TH positive neurons and Nissl positive neurons of mice pretreated with high dose EHT and lesioned with MPTP were not significantly different than those in saline injected animals. Indices of nigrostriatal terminal function were also higher with EHT treatment. Striatal dopamine level, which had dropped by 82 % with MPTP alone, was reduced by only 70 % in animals that had been treated with 0.1 % EHT ($p<0.05$) (Fig. 1d). Low dose EHT had no significant impact on this outcome measure. The increased dopamine turnover with MPTP intoxication, measured by the HVA/dopamine ratio, also decreased in mice treated with 0.1 % EHT ($p<0.05$) (Fig. 1e). Similar protection was observed by measuring striatal TH content with ELISA. Compared with 58 % decline with MPTP alone, 0.1 % EHT treatment was associated with only 43 % decline ($p<0.05$) (Fig. 1f). These observations collectively suggest a neuroprotective effect of EHT against the toxicity of MPTP.

The central effects of EHT could not be attributed to alterations in MPTP metabolism. In a separate experiment, mice were treated with two different doses of EHT (0.01 % and 0.1 %) or placed on control diet for 2 weeks prior to challenging them with a single dose of MPTP (30 mg/kg). Animals were sacrificed 90 min later, and striatal lysates subjected to HPLC. No significant difference in MPP^+ levels was found: control group (10.6±0.66 ng/mg tissue), low dose EHT treated group (10.03±0.14 ng/mg tissue) and high dose EHT treated group (10.93±0.10 ng/mg) (Fig. 1g).

EHT has an Anti-inflammatory Effect in the Mouse Brain

MPTP causes a robust neuroinflammatory response with microglial and astroglial activation in both midbrain and

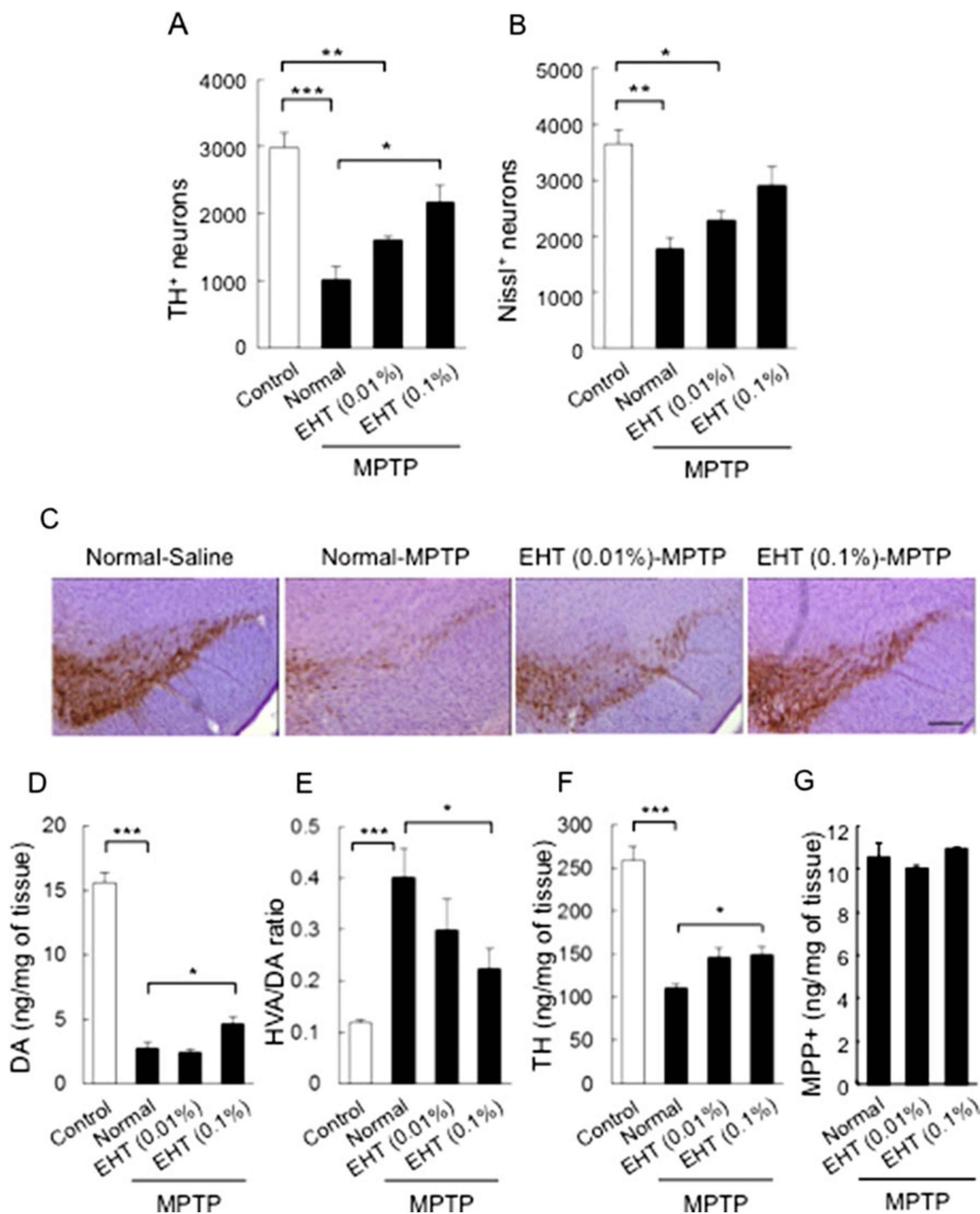

Fig. 1 EHT treatment attenuates MPTP-induced nigrostriatal dopaminergic neuronal damage. Mice were treated with EHT for 4 weeks and challenged with MPTP (10 mg/kg, every 2 h X4). Brains were analyzed 7 days post MPTP. **a**, **b** Stereological counting of TH-positive and cresyl-violet positive neurons in sections of the substantia nigra from one hemisphere. Normal diet-saline injections (control), n=4; normal diet-MPTP, n=3; EHT in diet (0.01 %)-MPTP, n=4; and EHT in diet (0.1 %)-MPTP, n=3. **c** Representative images of TH immunohistochemistry of midbrain sections. Scale bar=200 μm. **d** Striatal dopamine (DA) level measured by HPLC. Control (no EHT or MPTP), n=6; normal diet-MPTP, n=5; EHT (0.01 %)-MPTP, n=6; and EHT (0.1 %)-MPTP, n=5. **e** Dopamine turnover measured by HVA/dopamine ratio. **f** Striatal TH content measured by ELISA. Control, n=6; normal diet-MPTP, n=5; EHT (0.01 %)-MPTP, n=6; and EHT (0.1 %)-MPTP, n=5. **g** EHT treatment had no impact on MPTP metabolism in vivo. Mice were treated with two dose levels of EHT for 2 weeks prior to receiving a single dose of 30 mg/kg MPTP. Animals were sacrificed 90 minutes later (n=4 per group). MPP^+ levels were assessed by HPLC. *p<0.05, **p<0.01, ***p<0.001

striatum of the mouse model [19, 20], and markers of neuro-inflammation are also observed in PD brains [21]. This was also confirmed in our model of MPTP, particularly 3 days following lesion (Fig. 2a–d). As we had observed a reduction in inflammatory indicators with EHT treatment in an α-synuclein transgenic model of PD [6], we also assessed its efficacy in MPTP treated animals. Mice pretreated with EHT exhibited dose-dependent attenuation of both microglial and astrocytic activation, observed by immunohistochemistry with Iba-I and GFAP, respectively, achieving statistical significance with the higher dose of EHT (Fig. 2a–d).

EHT has Anti-oxidant Effect In Vivo

MPTP toxicity and neuroinflammation are known to be associated with oxidative stress resulting in depletion of reduced glutathione. To investigate additional properties of EHT in vivo, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was determined in the brains of mice challenged with MPTP or saline and pretreated with EHT or control diet. MPTP combined with control diet resulted in a significant 30 % decrease of GSH/GSSG ratio in the substantia nigra, whereas in animals pre-treated with

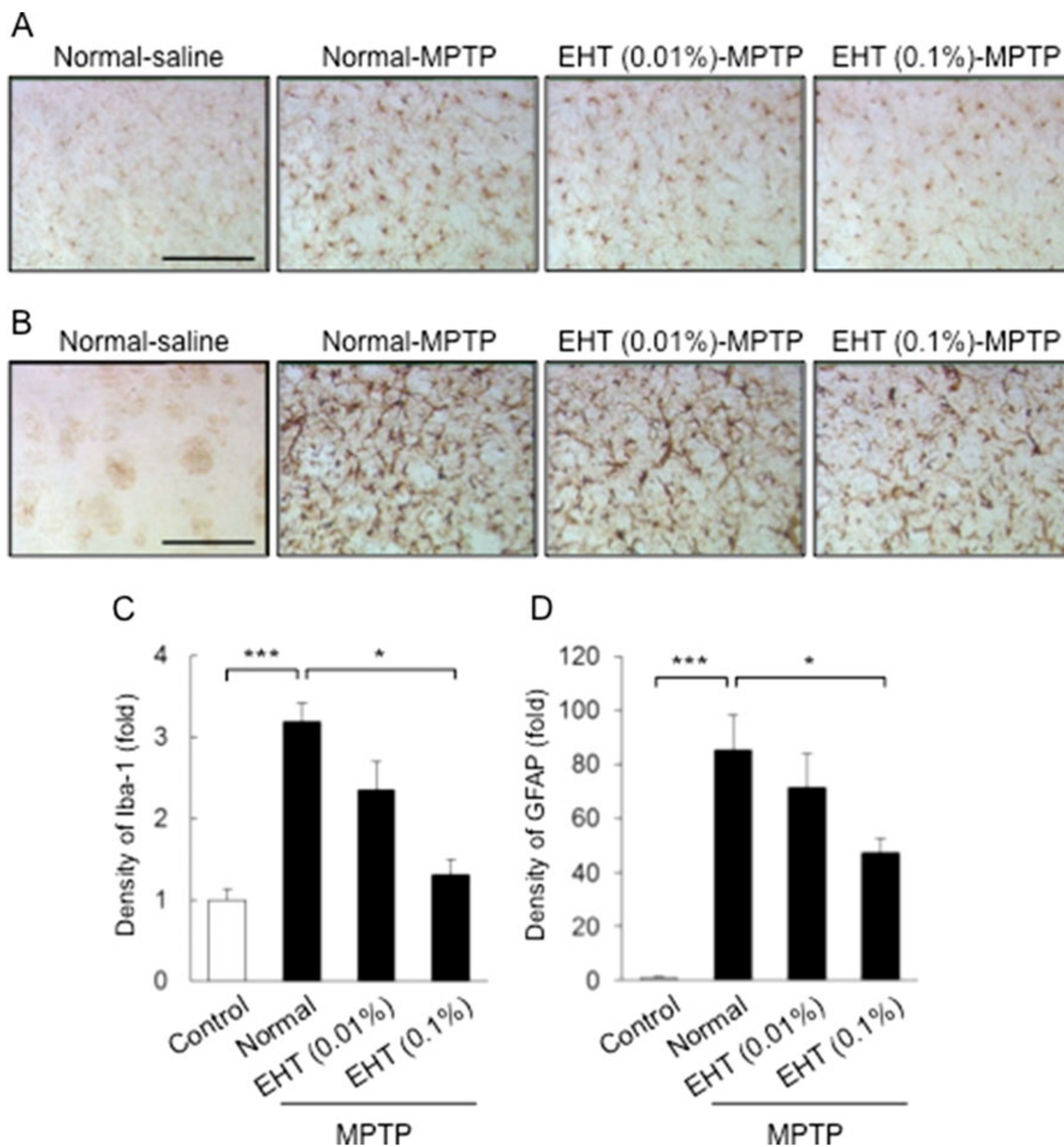

Fig. 2 EHT treatment attenuates MPTP-induced glial activation in the striatum. **a** EHT attenuates MPTP-induced microglial activation. Three days post MPTP, the increased microglia detected by Iba-1 immunoreactivity is significantly diminished in mice pretreated with EHT. **b** EHT attenuates MPTP-induced astrocytosis. Sections are stained with GFAP. **c** Quantification of images in panel A. **d** Quantification of images in panel C. Normal diet-saline, $n=4$; normal diet-MPTP, $n=5$; EHT (0.01 %)-MPTP and EHT (0.1 %)-MPTP, $n=5$. Scale bar=100 μm. *$p<0.05$, ***$p<0.001$

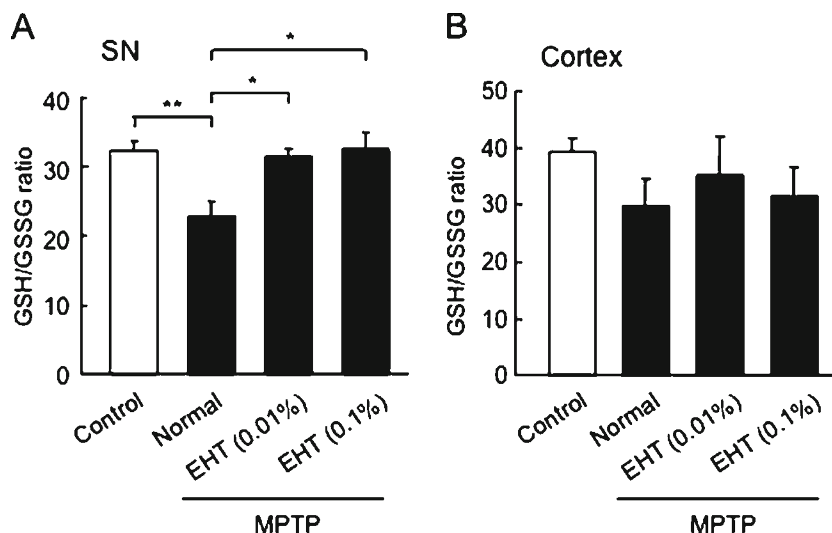

Fig. 3 EHT treatment prevents MPTP-induced depletion of reduced glutathione. **a** The decline in the ratio of reduced/oxidized glutathione (GSH/GSSG) 7 days following MPTP was completely prevented by EHT treatment in the substantia nigra (n=5 per group). **b** No significant change was seen in the cortex (n=3 per group). *p<0.05, **p<0.01

either dose level of EHT complete rescue of this index was observed (Fig. 3a). As expected based on the selective toxicity of MPTP in dopaminergic neurons, GSH/GSSG ratio did not change significantly in the cerebral cortex following MPTP challenge and was, therefore, not impacted by EHT treatment (Fig. 3b).

Consistent with this biochemical rescue of the oxidative stress following MPTP toxicity, EHT pre-treatment robustly attenuated MPTP-induced JNK activation (Fig. 4a and b). Compared with a 40-fold increase in phosphorylated-JNK (p-JNK) in the striatum of lesioned animals three days following MPTP administration, EHT pre-treatment was associated with a significantly weaker p-JNK response at both doses; a 0.01 % EHT containing diet resulted in only a 24-fold increase in p-JNK, while a 0.1 % EHT diet resulted in an 18-fold increase (p<0.01 comparing EHT-treated lesioned animals to control diet-fed lesioned animals).

EHT has Direct Anti-inflammatory Effects

To discern if the ability of EHT to repress the inflammatory response induced by MPTP in vivo may be due to a direct

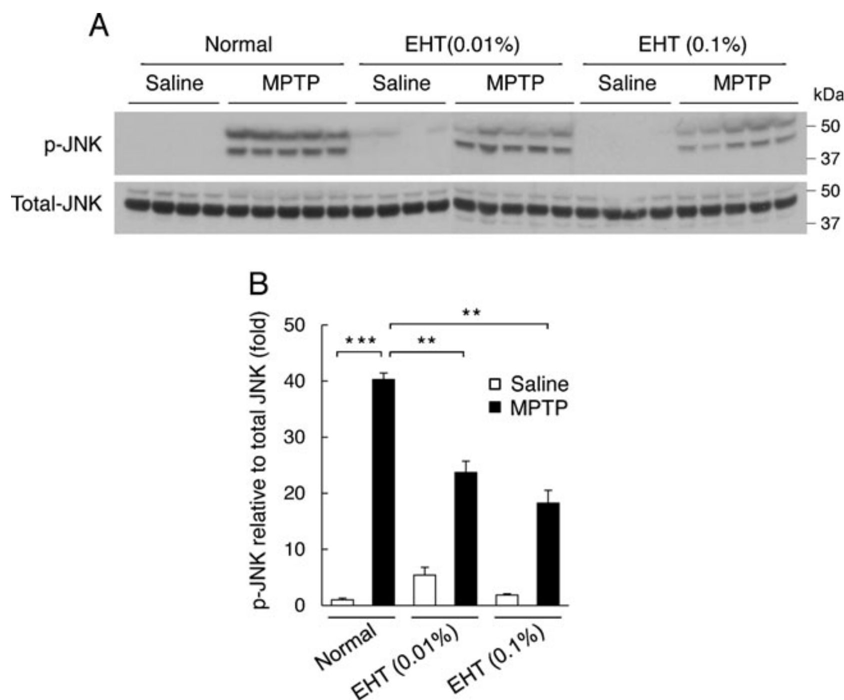

Fig. 4 EHT treatment attenuates MPTP-induced activation/phosphorylation of JNK. **a** Western blot for phosphorylated and total JNK in the striatum 3 days post-MPTP. **b** Quantification of p-JNK band intensities. **p<0.01, ***p<0.001

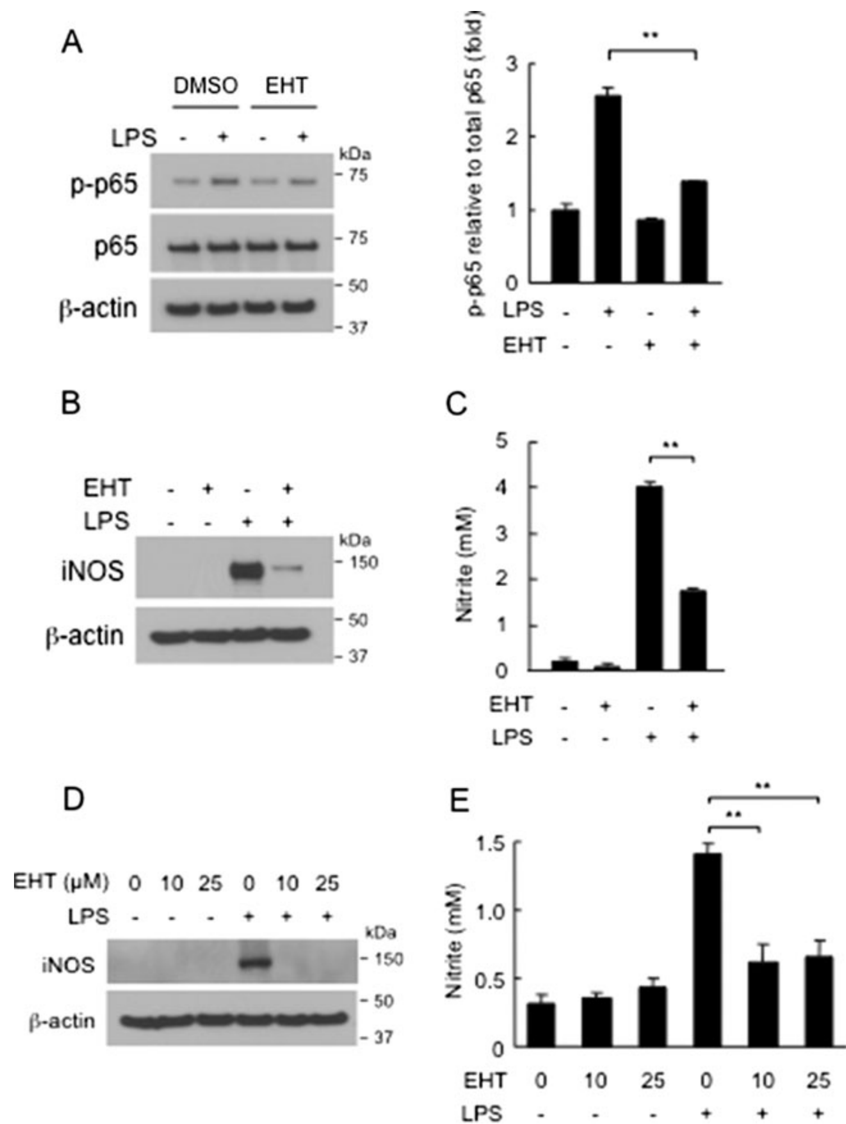

Fig. 5 EHT inhibits p65 activation, iNOS activation and nitric oxide production in primary microglia and astrocytes. **a** EHT inhibits LPS-induced p65 activation in primary microglia. Cells were pretreated with 10 μM EHT for 1 h and then stimulated with 1 μg/ml LPS for 15 min. Western blots were done for phospho-p65 (Ser536), p65, and β-actin. Bar graph depicts relative p-p65 level. ** $p<0.01$ ($n=3$). **b** EHT inhibits iNOS induction by LPS in primary microglia. Cells were pretreated with 10 μM EHT for 1 h and then stimulated with 1 μg/ml LPS for 24 h. **c** LPS induced nitric oxide production by microglia is significantly decreased by EHT. $n=6$. **$p<0.001$. **d** EHT suppresses iNOS expression in primary astrocytes. Cells were pretreated with 10 or 25 μM EHT for 1 h and then stimulated with 1 μg/ml LPS for 48 h. **e** EHT suppresses LPS induced nitric oxide production in astrocytes, $n=6$. ** $p<0.01$ relative to LPS treatment without EHT

anti-inflammatory response or is secondary to its neuroprotective effect, primary microglia and astrocytes in culture were pre-treated with EHT or vehicle and then exposed to the endotoxin lipopolysaccaride (LPS), which acts through TLR4 receptors. Compared to the robust activation of microglia with LPS alone indicated by increased phosphorylation/activation of p65 of NFκB (2.5-fold) (Fig. 5a), increased expression of iNOS detected by Western blotting (54-fold) (Fig. 5b), and increased nitric oxide production (21-fold) (Fig. 5c), adding EHT in the medium before LPS significantly repressed all these indices, resulting in their induction by only 1.4-fold, 9-fold and 9-fold, respectively (Fig. 5a–c). Similarly, EHT repressed LPS-induced iNOS induction and nitric oxide production in astrocytes (Fig. 5d, e). These findings indicate that EHT has a direct anti-inflammatory effect that could contribute indirectly to its neuroprotective activity in vivo.

EHT is an Anti-oxidant

The net anti-oxidant effect of EHT in the mouse brain demonstrated by its ability to prevent changes in the GSH/GSSG ratio (Fig. 3) and JNK activation (Fig. 4) could be due to a direct anti-oxidant effect or secondary to its anti-inflammatory effect or both. To test the first possibility, an in vitro assay was used with ABTS as a chromogen that changes into a colored radical in the presence of an oxidative agent and reduces to a colorless form when an anti-oxidant is added to the mixture. A range of concentrations of EHT was compared to two known anti-oxidants, ascorbic acid and Trolox, a water-soluble analog of vitamin E. This analysis showed that EHT is a much stronger anti-oxidant than Trolox and only slightly weaker than ascorbic acid (Fig. 6). EC_{50} concentrations: ascorbic acid=5.8±0.6 μM; EHT=23.9±7.8 μM; Trolox=372.1±59.5 μM.

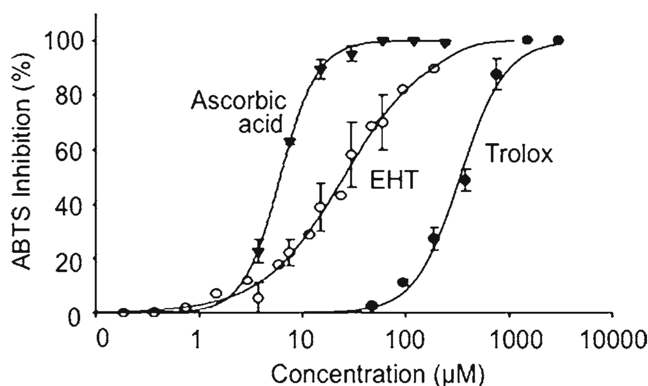

Fig. 6 EHT is a direct anti-oxidant. Compounds tested in the ABTS radical scavenging colorimetric assay: ascorbic acid (▼), EHT (○) and Trolox (●). Data shown are means ± SEM from three independent experiments

EHT Counters MPTP-induced PP2A Methylation Impairment and Confers Cytoprotection

We recently reported that EHT promotes the methylation of PP2A by directly inhibiting the interaction between methylated PP2A and its specific methylesterase [6]. PP2A is the master regulator of the cellular phosphoregulatory network [22] that controls signal transduction and apoptosis in virtually all vertebrate cell types [23–25]. We, therefore, assessed changes in PP2A methylation state associated with mitochondrial complex I inhibition and EHT treatment in SH-SY5Y neuroblastoma cells. Exposure to 3 mM MPP^+, the toxic metabolite of MPTP, for 18 h- resulted in a 2.1-fold increase in the level of demethylated PP2A determined by Western blot analysis ($p<0.01$). Total PP2A levels did not change significantly (Fig. 7a and b). On the other hand, pre-treatment of these cells with EHT at 10 μM and 25 μM significantly mitigated MPP^+ induced demethylation of PP2A (Fig. 7a and b). Parallel with these biochemical observations, EHT had a significant cytoprotective activity against the toxicity of MPP^+ in SH-SY5Y cells. Compared with 74 % cell death determined by LDH release following exposure to 3 mM MPP^+, pre-treatment with 10 μM and 25 μM EHT for 6 h was associated with only 54 % and 52 % cell death, respectively (Fig. 7c). One hour pre-treatment with 25 μM EHT was also protective (data not shown). Similar cytoprotection was verified with the MTT assay (Fig. 7d).

Discussion

This study demonstrates for the first time that MPTP toxicity in both the mouse brain and in cultured cells is attenuated by EHT, a component of coffee. The neuroprotective impact of EHT is associated with a marked anti-inflammatory effect demonstrated both in vivo and in cultured primary glial cells, antioxidant activity, and PP2A modulation. These observations add to our previous finding that chronic EHT administration mitigates the phenotype of α-synuclein transgenic mice [6], thus linking its efficacy in both genetically and toxin driven models of PD.

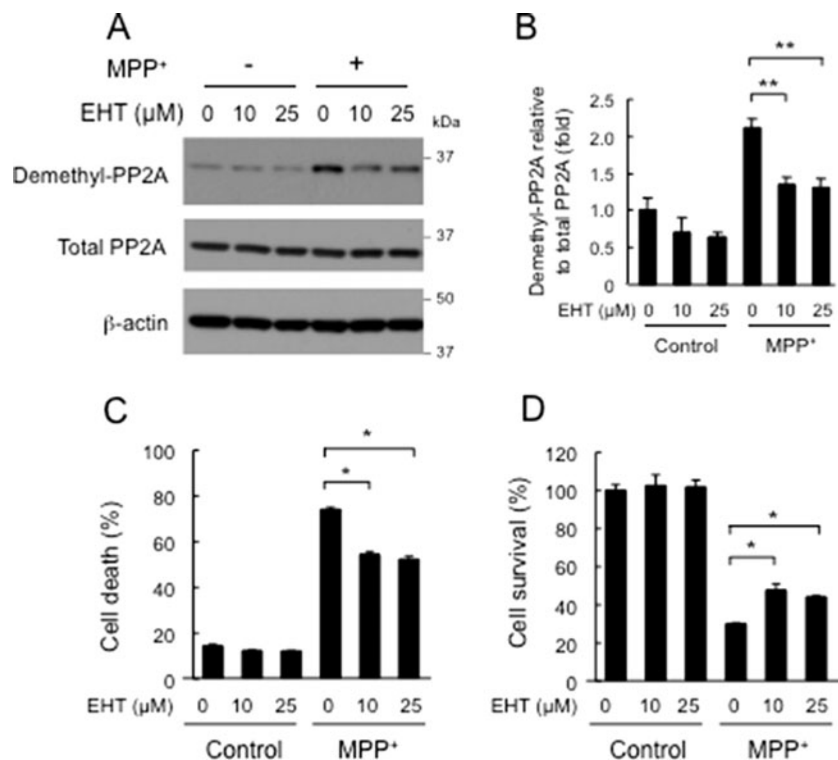

Fig. 7 EHT prevents MPP^+-induced demethylation of PP2A and cell death in SH-SY5Y cells. **a** EHT inhibits MPP^+-induced PP2A demethylation. Cells were pretreated with 10 μM EHT for 6 h and then stimulated with 3 mM MPP^+ for 18 h. Western blots were done for demethyl-PP2A, total PP2A, and β-actin. **b** Bar graph depicts relative demethyl-PP2A level. $^{**}p<0.01$ ($n=4$). **c** EHT suppresses MPP^+-induced cell death assessed by LDH release. Cells were pretreated with 10 μM EHT for 6 h and then stimulated with 3 mM MPP^+ for 18 h, $^{*}p<0.05$ ($n=6$). **d** EHT rescues MPP^+-induced diminished cell survival assessed by MTT assay. Cells were pretreated with 10 μM EHT for 6 h and then stimulated with 3 mM MPP^+ for 18 h, $^{*}p<0.05$ ($n=6$)

The mechanisms by which EHT confers neuroprotection are multiple. In addition to the direct protective role of EHT observed in cellular studies, its effects on inflammation appear to be key in its efficacy. Inflammation is a critical mechanism that can exacerbate neuronal damage in the brain with excessive release of cytokines and inflammatory mediators causing secondary damage [26]. Interestingly, EHT results in significantly reduced inflammation in both the MPTP model demonstrated in the present study and in α-synuclein transgenic mice [6]. It is conceivable that this repression of microglial and astrocytic cell activation could be secondary to the reduced drive for inflammation due to decreased neuronal death or diminished α-synuclein aggregation. However, the present demonstration of a robust inhibition of LPS-induced inflammatory markers in cultured primary microglia and astrocytes, including repression of NFκB activation, iNOS induction and increased nitric oxide production, indicates a direct anti-inflammatory effect of EHT. Thus, through repressing inflammation, EHT can indirectly protect neurons in models of neurodegeneration.

A second robust mechanism by which EHT can protect neurons in the MPTP model is through its antioxidant action. While its anti-inflammatory activity can certainly lead to less reactive oxygen species generation in vivo, the in vitro assay employed here indicates that it also has a direct antioxidant activity. EHT's activity is significantly more potent than a water-soluble analog of vitamin E and only slightly lower than vitamin C, both well characterized antioxidants. This action of EHT can contribute to its neuroprotective effects not only against the oxidative stress seen in the MPTP model [5, 27–29] but also in models of α-synuclein over-expression, which are associated with increased oxidative stress as well [30, 31].

The impact of EHT on PP2A methylation and activity is another mechanism contributing to neuroprotection. Consistent with reports that PP2A activity is inhibited by oxidative stress [32–35], MPP^+ challenge of cultured neuroblastoma cells caused increased demethylation of PP2A. On the other hand, EHT, which inhibits the interaction between methylated PP2A and its specific methylesterase PME-1 [6], prevents this modulation of PP2A and as a result is cytoprotective. Robust changes in PP2A methylation could not be demonstrated in the brains of mice challenged with MPTP and treated with EHT, likely because of the much more complex cellular mix in brain tissue where both neuronal degeneration and glial activation occur. We hypothesize that the glial response may overshadow the effect of MPTP on neuronal PP2A and mask the corrective action of EHT. Nevertheless, altered PP2A methylation by MPP^+ and its prevention by EHT demonstrated in cultured cells likely contributes to the changes in the inflammatory response seen in vivo as PP2A is intimately involved in inflammatory responses [36]. Additionally, normalization of PP2A function has been hypothesized to be relevant in relation to modulation of tyrosine hydroxylase function [37, 38].

The present findings suggest that EHT is protective in the MPTP model of PD through multiple specific mechanisms that are interrelated, including direct cytoprotective, anti-inflammatory and anti-oxidant activities. Conceivably, an underlying mechanism may involve PP2A, thus providing a critical central regulatory protein. Interestingly, PP2A methylation is decreased in the brains of Alzheimer's disease patients [39], another disorder for which consumption of coffee has been linked with reduced incidence [40]. It is intriguing to speculate that similar changes may be common to other neurodegenerative disorders such as PD. Accordingly, normalization of PP2A function is a potential target for neuroprotective drugs [41, 42].

The accumulating evidence about the protective effects of EHT in both the MPTP model and in α-synuclein transgenic mice [6] may inform why consumption of coffee is linked with reduced incidence of PD [7–10]. Caffeine has been extensively studied in an attempt to explain this role, in particular through its activity as an adenosine A2A receptor antagonist [43]. However, among patients with early PD, the amount of caffeine consumption does not impact the rate of progression of the disease [44], raising some question about the protective effect of just caffeine in coffee. EHT, therefore, provides a second component, acting through alternative mechanisms that can impact multiple PD relevant pathways.

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