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Review

Dopaminergic Neuron-Specific Oxidative Stress Caused by Dopamine Itself

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Oxidative stress, including the reactive oxygen or nitrogen species generated in the enzymatical oxidation or auto-oxidation of an excess amount of dopamine, is thought to play an important role in dopaminergic neurotoxicity. Dopamine and its metabolites containing 2 hydroxyl residues exert cytotoxicity in dopaminergic neuronal cells, primarily due to the generation of highly reactive dopamine and DOPA quinones. Dopamine and DOPA quinones may irreversibly alter protein function through the formation of 5-cysteinyl-catechols on the proteins. Furthermore, the quinone formation is closely linked to other representative hypotheses such as mitochondrial dysfunction, inflammation, oxidative stress, and dysfunction of the ubiquitin-proteasome system, in the pathogenesis of neurodegenerative diseases. Therefore, pathogenic effects of the dopamine quinone have recently focused on dopaminergic neuron-specific oxidative stress. In this article, we primarily review recent studies on the pathogenicity of quinone formation, in addition to several neuroprotective approaches against dopamine quinone-induced dysfunction of dopaminergic neurons.

Key words: dopamine quinone, quinoprotein, methamphetamine, Parkinson's disease, L-DOPA

Oxidative stress, including the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the oxidation of dopamine (DA), is thought to play a role in dopaminergic neurodegeneration in several pathological conditions.

It is well known that methamphetamine (METH) causes damage to striatal dopaminergic and serotonergic nerve terminals [1–3]. Various hypotheses regarding the mechanism responsible for METH-induced neurotoxicity has been proposed [4–15]. Several studies have demonstrated that endogenous dopamine (DA) plays an important role in mediating METH-induced neuronal damage [7, 10, 13, 14]. DA release and redistribution from synaptic vesicles to cytoplasmic compartments and consequent elevation of cytosolic oxidizable DA concentrations are thought to be related to DA terminal injury induced by METH exposure [7]. ROS such as superoxide (O_2^-) and hydroxyl radicals (·OH), generated by auto-oxidation of cytosolic free DA, appear to be involved in METH-induced dopaminergic neuronal damage [5]. However, the exact mechanism responsible for its striatum-specific neurotoxicity remains unclear.

In Parkinson's disease, L-DOPA therapy is a standard approach, as it is designed to replenish the loss of DA from dopaminergic neurons. Despite the marked benefits of L-DOPA, its long-term use may

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cause adverse effects, especially motor fluctuations such as the wearing-off phenomenon and dyskinesia, as well as psychiatric symptoms [16, 17]. In advanced Parkinson's disease, L-DOPA does not completely replenish DA because few dopaminergic neurons remain in the nigrostriatal pathway. The pathogenic effects of L-DOPA and DA have been well documented.

A number of studies have shown that oxidative stress, mitochondrial dysfunction, inflammation, and impairment of the ubiquitin-proteasome system play important roles in the METH-induced neurotoxicity [6, 18, 19] and the pathogenesis of sporadic and familial Parkinson's disease [20–27]. However, the mechanisms of dopaminergic neuron-specific cell loss have not been fully clarified. More recently, an additional factor, DA quinone formation, has been investigated. This article focuses on the role of quinone formation in neurotoxicity by reviewing studies of METH-induced neuronal damage and of L-DOPA-treated parkinsonian models. In addition, neuroprotective approaches against DA quinone-induced pathogenicity are also addressed.

Dopaminergic Neurotoxicity Related to Generation of Free Radicals

DA as a neurotransmitter is stable in the synaptic vesicle. However, when an excess amount of cytosolic DA exists outside of the synaptic vesicle in the damaged neurons, *i.e.*, after METH injection or L-DOPA treatment using parkinsonian models, DA is easily metabolized via monoamine oxidase (MAO) or by auto-oxidation to produce cytotoxic ROS, and then subsequent neuromelanin formation [28]. In the oxidation of DA by MAO, H_2O_2 and dihydroxyphenylacetic acid (DOPAC) are generated. In contrast, non-enzymatical and spontaneous auto-oxidation of DA produces O_2^- and reactive quinones [29]. Generated O_2^- is converted to H_2O_2 by superoxide dismutase (SOD) and O_2^- also reacts with nitric oxide radicals (NO·) to consequently generate peroxynitrite ($ONOO^-$) of RNS. In the dopaminergic neurons, where transition metals are abundant, H_2O_2 can react with metals, especially iron, to form the most cytotoxic radical, ·OH. Therefore, it is suggested that L-DOPA- or DA-induced cell death is mainly due to ROS generation in the auto-oxidation of L-DOPA and/or DA. Some reports have shown that DA or L-DOPA exert either neuroprotective or even no effects on ROS generation in some circumstances [30–32]. However, this excess DA- or L-DOPA-induced neurotoxicity mediated by the generation of free radicals has been reported in damaged neurons in many *in vitro* and *in vivo* studies [5, 33–35]. We have demonstrated that repeated administration of L-DOPA increases lipid peroxidation in the striatum of parkinsonian mice lesioned by intracerebroventricular injection of 6-OHDA [34].

Dopamine Quinone Formation as a Dopaminergic Neuron-specific Pathogenic Factor

In contrast to the general oxidative stress induced by ROS or RNS, the pathogenicity of quinone formation has recently received attention as dopaminergic neuron-specific oxidative stress [24, 35–38]. As mentioned above, when the DA neurons are damaged, an excess amount of cytosolic DA existing outside of the synaptic vesicle is spontaneously oxidized and produces O_2^- and reactive quinones such as DA quinones or DOPA quinones [29, 39]. DA quinones are also generated in the enzymatic oxidation of DA by cyclooxygenase (COX) as prostaglandin H synthase, lipoxygenase, tyrosinase and xanthine oxidase [29, 39–43]. These quinones are easily oxidized to the cyclized aminochromes: DA-chrome and DOPA-chrome, and then are finally polymerized to form melanin. Although ROS by the auto-oxidation of DA shows widespread toxicity not only in DA neurons but also in other regions, highly reactive DA quinone or DOPA quinone itself exerts predominant cytotoxicity in DA neurons and surrounding neural cells, since these quinones are generated from free cytosolic DA outside the synaptic vesicle.

The quinones generated from DA or L-DOPA exert cytotoxicity in or beside dopaminergic neurons by interacting with various bioactive molecules. The functional proteins that possess cysteine residues are thought to be the targets of these catechol quinones. The DA quinone and L-DOPA quinone conjugate with the sulfhydryl group of the amino acid cysteine, resulting predominantly in the formation of 5-cysteinyl-DA and 5-cysteinyl-DOPA, respectively [29, 44, 45]. The covalent modification of cysteine residues by DA quinone or DOPA quinone to form 5-cysteinyl-

catechols (5-cysteinyl-DA, -DOPA and -DOPAC) would irreversibly alter or inhibit protein function and consequently cause cytotoxicity, since the cysteine residues are often found at the active sites of functional proteins. This mechanism of neurotoxicity by the formation of 5-cysteinyl-catechols on protein is supported by evidence showing the generation of catechol quinones and the consequent formation of 5-cysteinyl-catechols in DA- and L-DOPA-induced cell loss of dopaminergic neuronal cells [46–48]. In particular, it is of interest that DA quinones generated in the brain covalently modify and inactivate tyrosine hydroxylase, a DA-synthesizing enzyme to subsequently form redox-cycling quinoprotein [49, 50]. The DA quinones also covalently conjugate with cysteine residues in the DA transporter (DAT) to consequently inhibit DA uptake [51]. More interestingly, LaVoie *et al.* have shown that DA covalently modifies parkin protein, in a gene mutation causing familial Parkinson's disease, to increase parkin insolubility and to inactivate its E3 ubiquitin ligase function [38]. Our previous study showed that the antisense knockdown of parkin causes apoptotic death of human dopaminergic SH-SY5Y cells accompanied by accumulation of DA-chrome generated from DA quinone [52]. Therefore, we suppose that parkin has a profile as a DA quinone-quenching molecule to prevent DA quinone-induced pathogenicity. The α-synuclein, which is a major component of the insoluble fibrils of Lewy bodies in patients with Parkinson's disease, is also one of the target proteins for DA quinone [53]. The soluble state of α-synuclein is converted to aggregate fibrils via transient formation of pathogenic protofibrils by its oligomerization [54]. Mutant α-synuclein (A30P), which is linked to familial autosomal dominant Parkinson's disease, enhances the rate of protofibril formation but inhibits the conversion of protofibrils to fibrils, thereby increasing toxic protofibrils [55]. This, in turn, leads to the destruction of synaptic vesicular membranes [56]. Conway *et al.* have found that DA quinone or L-DOPA quinone reacts with α-synuclein to form a quinoprotein DA quinone-α-synuclein adduct by coupling to tyrosine residues of α-synuclein and/or by nucleophilic attack on lysine residues forming a Schiff base, to consequently inhibit the conversion of toxic protofibrils to

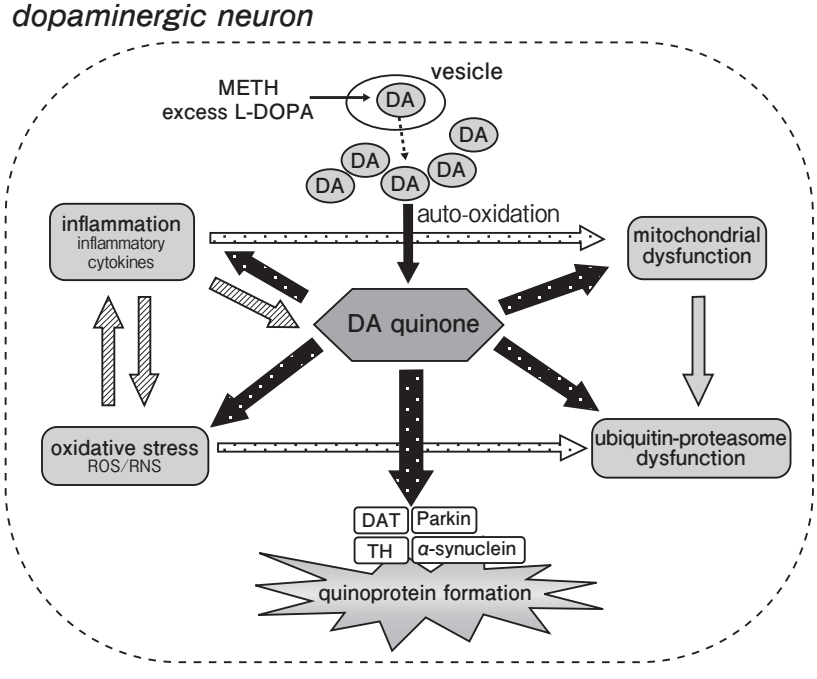

Fig. 1 Neurotoxicity of quinone formation as a dopaminergic neuron-specific oxidative stress, which closely links representative hypotheses, mitochondrial dysfunction, inflammation, oxidative stress, and impairment of the ubiquitin-proteasome system in the pathogenesis of dopaminergic neurodegeneration.

fibrils [53], although α-synuclein does not contain cysteine residues [50, 57]. These findings suggest that quinone formation by DA auto-oxidation plays an important role as a DA neuron-specific common toxic factor in the neurodegenerative processes by DA-related oxidative stress.

A number of studies have clarified that the cytotoxicity of quinone formation is closely linked not only to general oxidative stress but also to other representative hypotheses of METH neurotoxicity and pathogenesis in Parkinson's disease, including mitochondrial dysfunction, inflammation and proteasome impairment (Fig. 1). Quinone formation by DA oxidation reduces mitochondrial function and opens the mitochondrial permeability transition pores in the brain [58]. DHBT-1 (7-(2-aminoethyl)-3, 4-dihydro-5-hydroxy-2*H*-1, 4-benzothiazine-3-carboxylic acid), a further oxidation product of 5-cysteinyl-DA, irreversibly inhibits the activity of mitochondrial complex I [59]. Prostaglandin H synthase, which exerts both COX activity and peroxidase activity on the arachidonic acid cascade, catalyzes the production of prostaglandin G2 from arachidonic acid by its COX activity, and then converts prostaglandin G2 to prostaglandin H2/E2 by its peroxidase activity. In the presence of DA, oxidative reaction of DA to form DA quinone is coupled with the latter step. In other words, the DA quinone is generated not only in auto-oxidation of DA, but also by the enzymatic oxidation of DA by prostaglandin H synthase in a reaction mediated by H_2O_2 [40]. Recently, Kuhn *et al.* have shown that DA quinones activate microglia and induce neurotoxic genes in METH-induced toxicity [60]. These findings suggest that DA quinones are closely linked to inflammation and play an important role in the degeneration of DA neurons. Furthermore, apoptotic and selective dopaminergic cell death with formation of ubiquitin and α-synuclein-positive inclusion *in vitro* or *in vivo* induced by the proteasome inhibitor lactacystin or epoxomicin is suppressed by reducing endogenous DA, and is enhanced by treatment with L-DOPA or the monoamine oxidase inhibitor pargyline [61, 62]. In a related work, DA was found to induce proteasome inhibition in PC12 cells, and this inhibition was attenuated by glutathione (GSH), monoamine oxidase inhibitors, or a DA uptake inhibitor [63]. In addition, Zafar *et al.* have demonstrated that DA quinone has the ability to inhibit proteasomal activity [64]. These findings suggest a possible role of proteasome inhibition in the toxicity induced by high levels of DA or its quinone in the cytosol.

Involvement of Dopamine Quinone Formation in Methamphetamine-induced Neurotoxicity

METH-induced neurotoxicity is characterized by a long-lasting depletion of striatal DA as well as damage to striatal dopaminergic nerve terminals. Auto-oxidation of cytosolic free DA and consequent generation of ROS have been reported to be involved in METH-induced neurotoxicity in dopaminergic neurons [5–8, 65]. We have previously demonstrated that METH-induced dopaminergic neurotoxicity is regulated by quinone formation-related molecules [66]. METH exposure dose-dependently induced cell death in dopaminergic CATH. a cells. Levels of quinoprotein formation, which represents generation of DA quinones, also increased in a dose-dependent manner with METH treatment, coinciding with cell toxicity. Repeated METH injections have been reported to cause dopaminergic terminal loss shown as reduction of DAT-positive signals in the striatum of animals. In this study, we confirmed the reduction of DAT-positive signals in the striatum of BALB/c mice 1, 3, and 14 days after repeated METH injections (4 mg/kg x 4, i.p. with 2 h-intervals). Quinoprotein levels in the striatum were significantly increased 3 and 14 days after the repeated METH injections, coinciding with reduction of DAT signals. The induction of quinone reductase by butylated hydroxyanisol (BHA) treatment almost completely prevented METH-induced quinone generation, in parallel with the cell toxicity. These findings confirm that DA quinone formation is involved in METH-induced dopaminergic neurotoxicity. Furthermore, intracellular DA depletion with reserpine or α-methyl-*p*-tyrosine (α-MT) treatment significantly prevented the elevation of quinoprotein formation induced by METH exposure, suggesting that the reduction of endogenous DA could attenuate quinone toxicity. In fact, there are several reports showing that α-MT prevents the toxic effects of METH. Our present study has provided *in vitro* and *in vivo* evidence indicating that DA quinone formation by auto-oxidation of endogenous DA may play an important role in METH-induced neurotoxicity. Using vesicular monoamine transporter (VMAT)-2 knock-out

mice, Fumagalli *et al.* [8] showed that disruption of VMAT potentiates METH-induced neurotoxicity *in vivo* and pointed a greater contribution of intraneuronal DA redistribution rather than extraneuronal overflow. Furthermore, LaVoie and Hastings [9] demonstrated that increased intracellular DA oxidation is associated with METH neurotoxicity by measuring 5-cysteinyl-DA, a product of DA quinone bound to cysteinyl residue on protein, but not extracellular DA. These experimental findings indicate that DA quinone formation plays an important role in METH-induced neurotoxicity.

Role of Dopamine Quinone Formation in a Model of Parkinson's Disease

The toxicity of L-DOPA and DA has been well documented in many experiments [34, 35, 67–70], since we and others first demonstrated that L-DOPA itself can become a L-DOPA quinone radical and that the repeated administration of L-DOPA increases lipid peroxidation in the striatum of parkinsonian mice [71, 72]. However, there is some controversy regarding L-DOPA treatment-induced pathogenicity in patients with Parkinson's disease [73]. Long-term L-DOPA therapy causes several adverse effects only in parkinsonian patients and not in pathological conditions other than Parkinson's disease. Furthermore, the ELLDOPA study, which attempted to clarify whether L-DOPA is toxic and accelerates the progression of Parkinson's disease, showed that L-DOPA-treated patients had an increased rate of decline in striatal DA uptake (β-CIT) in SPECT, although patients treated with L-DOPA showed less clinical deterioration [74]. We previously revealed that repeated L-DOPA administration (50 mg/kg/day) for 7 days elevated striatal DA turnover (2.7-fold) and quinone generation (2.4-fold) specifically on the parkinsonian side, but not on the control side, of hemi-parkinsonian models [75–77]. Therefore, the excess amount of cytosolic DA outside of the synaptic vesicles after L-DOPA treatment may exert inhibitory effects on DA re-uptake function through dysfunction of DAT (decline of β-CIT uptake) by quinone generation, at least in the damaged dopaminergic nerve terminals. Furthermore, we recently revealed that DA agonists pergolide and pramipexole possess not only ROS/RNS-scavenging activities and GSH-increasing effects but also a quenching effect on DA-semiquinone radicals generated *in vitro* [75, 77]. In particular, pergolide dramatically prevents repeated L-DOPA administration-induced striatal quinone generation specifically on the lesioned side of hemi-parkinsonian models [77]. Regarding the specificity to the dopaminergic neuronal system and the clinical efficacy, DA agonists may be the most potential therapeutic reagents against DA quinone-induced dysfunction of DA uptake.

Neuroprotective Molecules and Drugs against Dopamine Quinone Toxicity

The intrinsic antioxidative molecules protect brain tissue from the DA quinone- or L-DOPA quinone-induced neurotoxicity. DA- or L-DOPA-induced formation of these quinones, the consequent quinone-induced formation of 5-cysteinyl-catechols on functional proteins [29, 44, 45], and the death of dopaminergic cells [46–48] are markedly prevented by the addition of superoxide dismutase (SOD), GSH, or cysteine, which competes with the sulfhydryl group of cysteine on proteins conjugating with DA or DOPA quinones, but not by the addition of catalase [35, 46, 47, 50, 69, 71]. We previously revealed the neurotoxic properties of DA- and L-DOPA-related compounds in human neuroblastoma SH-SY5Y cells to generate DA- or DOPA-semiquinone radicals, which are consequently converted to toxic quinones, and that cell death and the formation of these semiquinone radicals induced by DA or L-DOPA are markedly prevented by the addition of Cu/Zn-SOD or GSH, but not by the addition of catalase [71]. The SOD can act as a superoxide: semiquinone oxidoreductase to prevent quinone formation [78]. Furthermore, by conjugating with DA quinone, GSH prevents DA quinone from binding to the sulfhydryl groups of cysteine on proteins, as described above. Therefore, the protective effects of SOD and GSH against DA- or L-DOPA-induced cytotoxicity may be the result of these antioxidants' quinone-quenching activities. The involvement of quinone formation in DA- or DOPA-induced cytotoxicity is also supported by our recent study, in which overexpression of Cu/Zn-SOD protects SH-SY5Y cells against DA-induced cytotoxicity accompanied by an increase in their GSH level [71]. If DA-related neuronal death is caused by the genera-

tion of general ROS or RNS, the cytotoxicity of DA would be prevented by antioxidants such as ascorbic acid and α-tocopherol. However, ascorbic acid and α-tocopherol have lesser or no effects against DA-induced cell death in PC12 cells, while cell death is markedly inhibited by the thiol-containing compounds GSH, *N*-acetylcysteine, and dithiothreitol [79]. These findings suggest that intrinsic thiol molecules including GSH prevent DA quinone from binding to the sulfhydryl groups of cysteine on functional proteins by their conjugation with DA quinone [35, 40]. However, a large amount of GSH or its precursor *N*-acetylcysteine is required in the peripheral administration to maintain an effective concentration against quinone toxicity in the brain, since these small peptide drugs are easily broken down before they cross the blood-brain barrier.

Treatment with BHA, dimethyl fumarate, or *tert*-butylhydroquinone, each of which up-regulates the activity of NAD(P)H: quinone oxidoreductase (NQO: DT-diaphorase), protects against cell death related to quinone formation [36, 80–82]. In particular, dimethyl fumarate increases not only the activity of NQO: DT-diaphorase but also total intracellular GSH and the activities of GSH-*S*-transferase and GSH reductase [82] to reduce the cytotoxicity associated with DA quinone formation. Therefore, some reagents that activate NQO may potentially inhibit DA quinone-induced neurotoxicity. As described above, we have shown that pretreatment of dopaminergic CATH.a cells with NQO-1 inducer, BHA, significantly and dose-dependently blocks METH-induced elevation of quinoprotein, and ameliorates METH-induced cell death [66].

Moreover, other potential therapeutic drugs against quinone toxicity are nonsteroidal anti-inflammatory drugs (NSAIDs) that exert anti-inflammatory and neuroprotective effects against pathogenesis and dopaminergic neurodegeneration in parkinsonian patients. NSAIDs have inhibitory effects against prostaglandin H synthase, which oxidize DA to form DA quinone. The NSAIDs indomethacin and aspirin inhibit this prostaglandin H synthase-mediated oxidation of DA [83]. In animal experiments, several NSAIDs have been found to protect against dopaminergic cell loss in MPTP-treated parkinsonian models [84–86]. In addition, the reduction of nigral DA neurons by MPTP injection was prevented in COX-2 knockout mice or by treatment with a COX-2 inhibitor [87]. Furthermore, it is indicated that NSAIDs have protective effects against METH-induced dopaminergic cell loss in our reports [88]. Therefore, NSAIDs that may suppress the formation of DA quinones by inhibiting prostaglandin H synthase could be effective in the treatment of not only Parkinson's disease but also METH neurotoxicity, through their anti-inflammatory action and their reduction of catechol quinone-induced cytotoxicity [88, 89].

The melanin-synthetic enzyme tyrosinase in the brain may rapidly oxidize excess amounts of cytosolic DA, thereby preventing slowly progressive cell damage by auto-oxidation of DA [35]. In our previous report, we demonstrated that tyrosinase inhibition and transfection of antisense tyrosinase cDNA markedly reduced cell viability, increased intracellular DA, and enhanced DA-induced cell death in CATH.a cells [90], suggesting that the dysfunction of tyrosinase produces cell death by increasing intracellular DA levels and the consequent gradual auto-oxidation of DA to generate toxic ROS and reactive quinones, including DA quinone. Furthermore, we have shown the protective effects of tyrosinase against METH-induced dopaminergic neurotoxicity *in vitro* and *in vivo* [66]. In particular, the reduction of striatal DAT induced by the METH injection was dramatically aggravated in the tyrosinase-null mice, which showed higher quinoprotein levels, compared with that in the wild-type mice. These results suggest that tyrosinase plays a protective role against METH-induced dopaminergic neurotoxicity in neuronal cells by regulating quinone formation.

Cysteine-rich metal-binding proteins, metallothionein (MT)-1 and -2 are considered by some investigators to have a therapeutic potential based on their neuroprotective role [91, 92]. MTs are a family of low-molecular weight, cysteine-rich (30% of the protein), ubiquitous, and inducible intracellular proteins that bind to heavy metals such as zinc, copper, and cadmium, participating in metal homeostasis and detoxification [93]. MTs help to regulate metal homeostasis in the brain as well as exerting neural protective functions in various pathological and inflammatory conditions [93, 94]. Attention has been focused on MTs as radical scavengers because of their abundant thiol groups, which participate exclusively in the formation of metal-thiolate clusters [95–98].

Recently, we have reported that MT quenches DA semiquinones *in vitro*, and that MT protects against DA quinone neurotoxicity *in vitro* and *in vivo* [99].

Concluding Remarks

In conclusion, quinone formation by auto-oxidation of DA may play an important role as a DA neuron-specific common pathogenic and/or degenerative factor not only in METH neurotoxicity but also in dysfunction of dopaminergic neurons of the parkinsonian model treated with L-DOPA, because DA quinone is closely linked to oxidative stress, mitochondrial dysfunction, inflammation, and proteasome impairment (Fig. 1). Furthermore, we reviewed some neuroprotective molecules and drugs against quinone-induced pathogenicity. Enhancing activities of quenching or inhibitory properties against quinone formation would provide a novel neuroprotective approach to prevent dopaminergic neurodysfunction and/or neurodegeneration.

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