



Neurodegenerative Etiology of Aromatic L-Amino Acid Decarboxylase Deficiency: a Novel Concept for Expanding Treatment Strategies

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Abstract

Aromatic l-amino acid decarboxylase deficiency (AADC-DY) is caused by one or more mutations in the *DDC* gene, resulting in the deficit in catecholamines and serotonin neurotransmitters. The disease has limited therapeutic options with relatively poor clinical outcomes. Accumulated evidence suggests the involvement of neurodegenerative mechanisms in the etiology of AADC-DY. In the absence of neurotransmitters' neuroprotective effects, the accumulation and the chronic presence of several neurotoxic metabolites including 4-dihydroxy-L-phenylalanine, 3-methyldopa, and homocysteine, in the brain of subjects with AADC-DY, promote oxidative stress and reduce the cellular antioxidant and methylation capacities, leading to glial activation and mitochondrial dysfunction, culminating to neuronal injury and death. These pathophysiological processes have the potential to hinder the clinical efficacy of treatments aimed at increasing neurotransmitters' synthesis and or function. This review describes in detail the mechanisms involved in AADC-DY neurodegenerative etiology, highlighting the close similarities with those involved in other neurodegenerative diseases. We then offer novel strategies for the treatment of the disease with the objective to either reduce the level of the metabolites or counteract their prooxidant and neurotoxic effects. These treatment modalities used singly or in combination, early in the course of the disease, will minimize neuronal injury, preserving the functional integrity of neurons, hence improving the clinical outcomes of both conventional and unconventional interventions in AADC-DY. These modalities may not be limited to AADC-DY but also to other metabolic disorders where a specific mutation leads to the accumulation of prooxidant and neurotoxic metabolites.

Keywords Antioxidants · Inborn error of metabolism · Methylation status · Neuroinflammation · Parkinson's disease · Rare genetic disease · Sulfur-containing compounds

Abbreviations

AADC	Aromatic L amino acid decarboxylase	DHLA	Dihydrolipoic acid
AAV	Adeno-associated virus	DOPAL	3,4-Dihydroxyphenylacetaldehyde
ALA	α -Lipoic acid	DT	Deficient
ALS	Amyloid lateral sclerosis	DY	Deficiency
AD	Alzheimer's disease	EPO	Erythropoietin
BBB	Blood-brain barrier	GDNF	Glial-derived neurotrophic factor
BHMT	Betaine-homocysteine methyltransferase	GFAP	Glial fibrillary acidic protein
CAT	Cysteine aminotransferase	GSH	Glutathione
CBS	Cystathionine β -synthase	GSSG	Oxidized glutathione
COMT	Catechol- <i>O</i> -methyl-transferase	HCA	Homocysteic acid
CSE	Cystathionine γ -lyase	Hcy	Homocysteine
EP	Epinephrine	5-HIAA	5-Hydroxyindoleacetic acid
DAMPs	Damage-associated molecular pattern	H ₂ S	Hydrogen sulfide
		5-HT	Serotonin
		5-HTP	5-Hydroxytryptophan
		HMGB	High mobility group protein box
		HVA	Homovanillic acid
		icv	Intracerebroventricular
		L-dopa	3,4-Dihydroxy-L-phenylalanine

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MAO	Monoamine oxidase
MAT	Methionine adenosyl transferase
mDNA	Mitochondrial DNA
MHPG	Methoxy-4-hydroxyphenylglycol
3-MP	3-Mercaptopyruvate
MS	Methionine synthase
MST	3-Mercaptopyruvate sulfurtransferase
MT	Methyl transferase
MTHF	N-5-methyl tetrahydrofolate
NAC	N-acetyl-cysteine
NaHS	Sodium hydrogen sulfide
NEP	Norepinephrine
NMDA	N-Methyl-D-aspartate
Nrf2	Nuclear factor erythroid-2-related factor2
6-OHDA	6-Hydroxydopamine
3-OMD	3-O-methyl dopa
PD	Parkinson's disease
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SN	Substantia nigra
SOD	Superoxide dismutase
(TGF)- α	Transforming Growth Factor
Trx	Thioredoxin
VD3	Vitamin D3

Pathophysiology of AADC-DY

Aromatic l-amino acid decarboxylase deficiency (AADC-DY) is a rare autosomal recessive neurometabolic disorder caused by one or more mutations in the *DDC* gene, leading to a deficit in the AADC, the final enzyme in the biosynthesis of the monoamine neurotransmitters, known to catalyze the decarboxylation of l-3,4-dihydroxyphenylalanine (L-dopa) to dopamine and of L-5-hydroxytryptophan (5-HTP) to serotonin [1]. The deficit in dopamine synthesis can affect the three main brain dopaminergic pathways: the nigro-striatal pathway, the meso-limbic pathway, and the meso-cortical pathway, leading to motor and cognitive dysfunctions [2], whereas serotonin deficit has a significant impact on neurodevelopment with adverse effects on mood and behavior [3].

The affected individuals often present with high levels of metabolites upstream from the metabolic defect such as L-dopa, 3-O-methyl dopa (3-OMD), and 5-HTP, and decreased levels of metabolites downstream from the metabolic defect, including homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), norepinephrine (NEP), epinephrine (EP), and 5-hydroxyindoleacetic acid (5-HIAA). As a result, AADC-deficient (DT) subjects experience neurodevelopmental delay, movement disorders; including oculogyric crises, hypotonia, dystonia, and

hypokinesia; and autonomic dysregulations including sleep disorder and mood disturbances [4, 5]. These clinical symptoms are often manifested during the first months of life, although there is a considerable variability in the clinical presentation and severity of symptoms, depending in part to the degree of the impact of the enzyme deficiency on the synthesis of each of the 4 neurotransmitters, serotonin, dopamine, and its catecholamine derivatives, NEP and EP [4]. The disease has a high prevalence in Taiwan due to the Chinese DDC founder mutation [6].

Treatment of AADC-DY

The treatment modalities of AADC-DY, either the conventional or the unconventional, follow closely that of Parkinson's disease (PD), a disease involving a selective loss of nigrostriatal dopaminergic neurons, where disease progression and cell death leads to the loss of brain AADC enzyme activity [7].

Dopamine agonists are the first line of treatment of AADC-DT patients, with the objective to increase brain monoamine neurotransmitter production, and more common are the ergot-derived dopamine agonists such as bromocriptine and pergolide [8]. Although these drugs have been shown to improve some of the clinical symptoms of the AADC-DY, they are known to cause fibrotic reactions at the level of heart, lung, and retroperitoneal space; also, such reactions are less likely with the non-ergot-derived dopamine agonists [9]. Bromocriptine, the most prescribed dopamine agonist, is an antidiabetic drug able to reduce plasma glucose and hepatic glucose productions [10]. Studies in rodents show that these effects of bromocriptine are accompanied by a concurrent significant reduction in the serotonergic and noradrenergic activities, indicated by reduced levels of the metabolites 5HIAA, MPG, and HVA in the ventromedial hypothalamus [11]. Therefore, the use of bromocriptine in AADC-DT subjects, who have neurotransmitters' deficit and are often hypoglycemic, would likely be contraindicated. Similarly, the treatments with L-dopa and 5-HTP are contraindicated since AADC-DT subjects are unable to metabolize the high level of endogenous L-dopa and 5-HTP due to AADC enzyme deficiency, leading to poor clinical responses [4, 8].

An additional treatment modality for AADC-DY is the use of monoamine oxidase (MAO) inhibitors, with the objective to minimize dopamine and serotonin breakdowns. This regimen is often combined with dopamine agonists and or pyridoxine/pyridoxal phosphate, the AADC enzyme cofactor. Anticholinergics, melatonin, benzodiazepines, and α_2 adrenergic agonists are among less common modalities to treat the AADC-DY clinical symptoms [4, 8, 12]. Although these different regimens either singly or combined

have shown some clinical efficacy, most have unwanted side effects and the overall clinical outcomes of these limited treatment modalities remain poor [12, 13]. The non-pharmacological treatment of AADC-DT subjects includes ongoing physical, occupational, and speech therapies [8].

The newer treatment of AADC-DY has the objective to correct the AADC-related genetic defect, increasing brain dopamine production, using the recombinant adeno-associated viruses (AAV), the vector for the delivery of the functional DDC gene. Gene transfer in AADC-DY is based on earlier studies in PD patients receiving bilateral intraputamenal infusion of the non-pathogenic, low immunogenic AAV2 vector containing the human (h) DDC gene as a mean to enhance the AADC enzyme activity in the post-synaptic cells that express dopamine receptors, thus promoting the local metabolism of the administered L-dopa [14]. These studies reported improvements in the mean scores on the Unified Parkinson's Disease Rating Scale [15, 16], as well as increases in the short- and long-term AADC enzyme activities [16].

The studies of AAV2 vector-mediated intraputamenal hDDC gene transfer in AADC-DY were conducted by Hwu and colleagues [17, 18] in Taiwan, reporting improvements in motor functions and increase in brain dopamine levels, indicated by enhanced putamen's uptake of the AADC tracer 6-[(18) F]fluorodopa on PET imaging; these improvements were sustained at a 5-year follow-up [19]. A modification to the AAV2-mediated gene transfer in AADC-DT subjects was carried out by Pearson et al. [20], selecting the mid-brain, instead of putamen, as the anatomical site, based on the notion that dopaminergic neurons in the midbrain and their axonal projections remain intact in the AADC-DT subjects [21]. The investigators reported increased dopamine in the site of gene delivery, in both putamen and caudate nucleus due to anterograde axonal transport via the intact nigrostriatal pathway, and improvements in both motor and non-motor functions. Based on these clinical outcomes, the AAV2-mediated functional hDDC gene transfer has been recently approved in Europe for the treatment of ≥ 18 -month-old subjects with confirmed AADC-DY based on clinical, molecular, and genetic tests [22]. Nevertheless, the shape of the skull bones, if unsuitable for stereotactic surgery, and a pre-existing immunity to AAV2 limit the applicability of the AAV2-mediated gene transfer for AADC-DY treatment [18].

Accumulated Metabolites Induce Neuroinflammation

Among the underlying pathologies, which are overlooked in AADC-DY, are oxidative stress and inflammation, two processes that can lead to neuronal injury and death. In the following paragraphs, we describe the role of various

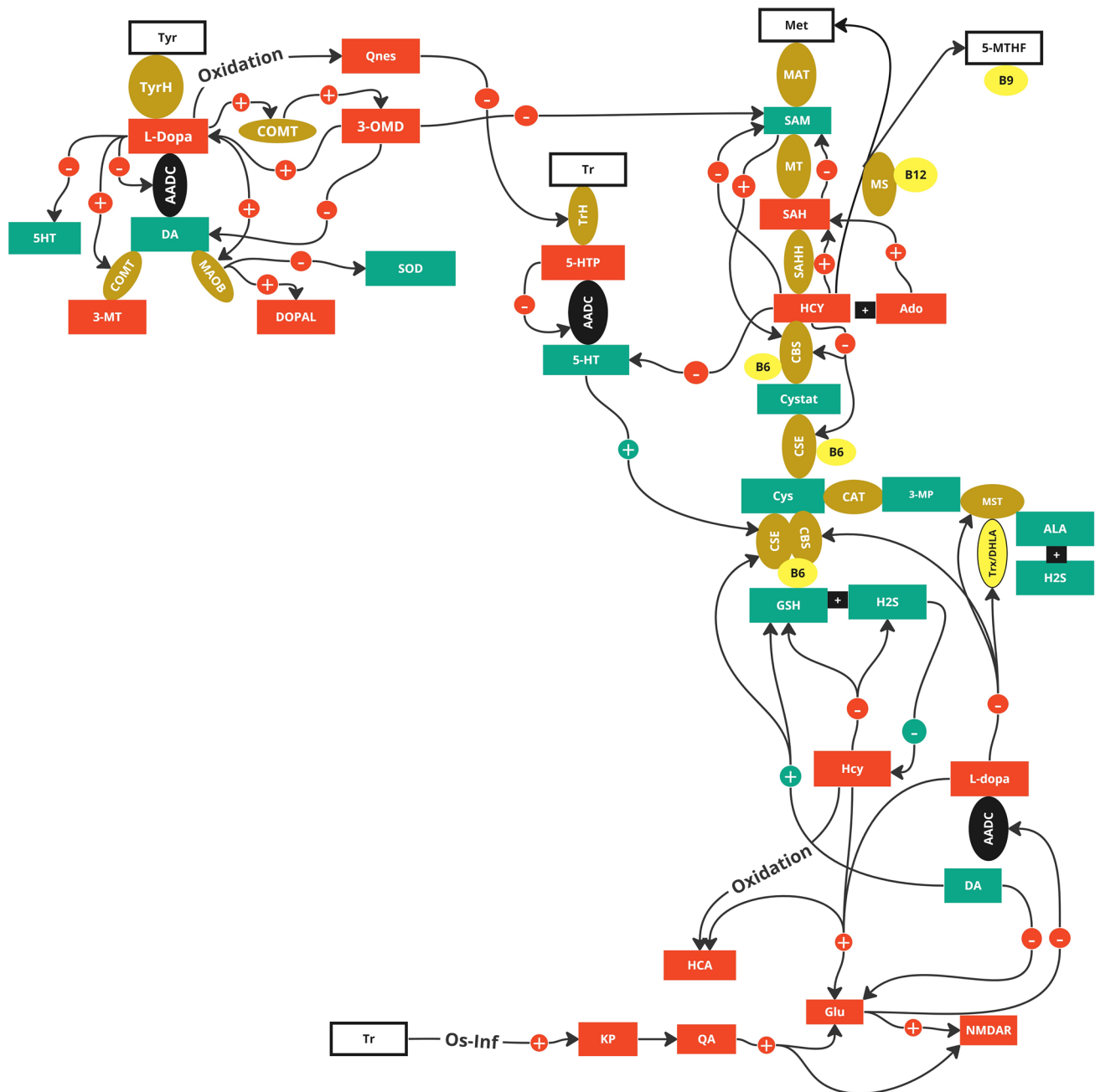
metabolites and their potential to induce, either singly or in a synergistic manner, oxidative stress, glial activation, and neuroinflammation, thus promoting neuronal injury and death. We will highlight the similarities between those and mechanisms involved in other neurodegenerative diseases. This new concept of AAD-DY as a neurodegenerative disease would likely open a path to novel treatment strategies, that when used early in the course of the disease, would likely minimize neuronal injury, preserving the functional integrity of neurons, hence improving the clinical outcomes of both conventional and unconventional AADC-DY interventions. The effects of accumulated metabolites and the pathways to neuroinflammation are depicted in Fig. 1.

Among metabolites known to be upregulated in AADC-DY are L-dopa and its major metabolite 3-OMD, which is produced by the enzymatic action of catecholamine-o-methyltransferase (COMT) enzyme. The increase in the levels of the two metabolites is the result of the lack of feedback inhibition, which is normally exerted by the end-product, dopamine, on tyrosine hydroxylase enzyme. Since dopamine is not produced, a high level of L-dopa accumulates, which is subsequently metabolized to 3-OMD, in a reaction that consumes the methyl donor, *s*-adenosylmethionine (SAM). The accelerated rate of *o*-methylation leads to the enhanced production of *s*-adenosylhomocysteine (SAH) which is rapidly converted to homocysteine (Hcy) and adenosine.

3-Methyldopa (3-OMD)

The major metabolite, 3-OMD, is produced from L-dopa in many organs including the brain, peripheral tissues, and the blood [25]. Comparing the level of 3-OMD and L-dopa, the CSF level of 3-OMD in AADC-DT subjects is many times higher than that of L-dopa [4, 24]. CSF levels of L-dopa in AADC-DT subjects range between 2 and 549 nm (reference range < 25 nm) whereas 3-OMD levels range between 54 and 4293 nm (reference range < 100 nm) [42]. There are several reasons for the higher CSF levels of 3-OMD compared to the levels of L-dopa.

The 1st reason is related to the differences in the biological half-life of the two metabolites. The half-life of L-dopa is about 1 h, that of 3-OMD is 15 h [43]. As a result, the ratio of 3-OMD to L-dopa concentration can be as high as 14:1 [44], leading to brain 3-OMD accumulation in AADC-DT subjects, with the potential to exert oxidative stress and cytotoxicity [45]. In PD patients, treated with L-dopa, the high concentration of plasma 3-OMD [46, 47] and the ratio of 3-OMD to L-dopa correlate with both dyskinesia [48] and poor response to L-dopa therapy [49]. In addition, the high CSF levels of 3-OMD correlate with the wearing-off phenomenon post-L-dopa therapy in PD [50], suggesting an association between 3-OMD levels and motor dysfunction. Although 3-OMD is a marker of AADC-DY diagnosis



[51, 52], there are no studies investigating the relationship between 3-OMD or 3-OMD/L-dopa and the heterogeneity in the AADC-DY clinical presentation; or heterogeneity in response to treatment, whether conventional or post-AAV2-mediated gene transfer.

The 2nd reason for the higher CSF 3-OMD levels in AADC-DY is related to COMT enzyme characteristics, the activity of which is substrate-dependent. COMT activity is enhanced due to the excess L-dopa availability [23], leading to accelerated *o*-methylation rate of L-dopa to 3-OMD, through consumption of SAM. Although L-dopa initially increases SAM levels due to augmented expression and

activity of methionine adenosyl transferase (MAT) [23], the enzyme that produces SAM, the significant increase in the rate of *o*-methylation subsequently decreases SAM levels and increases brain SAH [25, 26]. The SAM/SAH ratio is known to be an indicator of cellular methylation correlating with glutathione/oxidized glutathione (GSH/GSSG) ratio [53], an indicator of cellular antioxidant capacity and redox homeostasis, associated with neurodegeneration [54]. The increased rate of *o*-methylation can also accelerate dopamine methylation rate by COMT enzyme and the formation of the metabolite 3-methoxytyramine [23], which has low biological activity as a result of reduced

Fig. 1 Adverse effects of upregulated metabolites in the brain of AADC-DT subjects. The deficit in dopamine to exert inhibitory effect on tyrosine hydroxylase enzyme leads to the accumulation of brain L-dopa and subsequent activation of the COMT enzyme [23], enhancing 3-OMD [4, 24], which in turn leads to the increase in the rate of *o*-methylation with the subsequent reduction in SAM and the accumulation of Hcy, SAH [25, 26], and HCA [27]. These processes enhance excitotoxicity and reduce the level of the antioxidant precursor, cysteine [28]. In addition, metabolite-induced oxidative stress and excitotoxicity, singly or in synergy, reduce the activity of BCE [29], CSE [30], and MST [31] enzymes, as well as reduce the activity of the cofactor Trx in neurons [32], all of which are involved in the production of antioxidants and neuroprotective agents such as H2S, GSH, ALA, and SOD. The metabolite-induced prooxidant environment and the reduced cellular antioxidant capacity leads to glial activation [33–36], mitochondrial dysfunction [37], and neuroinflammation, promoting neuronal injury and death. Oxidative stress brought about L-dopa and Hcy has the potential to upregulate the KP pathway [38, 39], with the generation of QA which exerts neurotoxic effects via both increase in glutamate release and stimulation of NMDA receptors [40]. These many adverse effects of metabolites, combined with their ability to inhibit AADC enzyme activity [41], would have the potential to impede the clinical efficacy of treatments aiming to enhance neurotransmitters' synthesis and or functions. The red highlights present the upregulated and the green highlights present the downregulated metabolites and products. The brown highlights present the enzymes, and the yellow highlights present the cofactors. Abbreviations: AADC, aromatic L amino acid decarboxylase; Ado, adenosine; ALA, α -lipoic acid; CAT, cysteine aminotransferase; CBS, cystathionine β -synthase; COMT, catechol-*O*-methyl-transferase; CSE, cystathionine γ -lyase; Cys, cysteine; Cyst, cystathionine; DA, dopamine; DHLA, dihydrolipoic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; Glu, glutamine; GSH, glutathione; HCA, homocysteic acid; Hcy, homocysteine; H2S, hydrogen sulfide; 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; Ketog, ketoglutarate; KP, kynurenine pathway; L-dopa, 3,4-dihydroxy-L-phenylalanine; MAO, monoamine oxidase; MAT, methionine adenosyl transferase; Met, methionine; 3-MP, 3-mercaptopyruvate; MS, methionine synthase; MST, 3-mercaptopyruvate sulfurtransferase; MT, methyl transferase; MTHF, N-5-methyl tetrahydrofolate; 3-MT, 3-methoxytyramine; NMDAR, *N*-methyl-D-aspartate receptor; 3-OMD, 3-*O*-methyl-dopa; Os-Inf, oxidative stress-inflammation; QA, quinolinic acid; Qnes, quinones; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; SOD, superoxide dismutase; Tr, tryptophan; TrH, tryptophan hydroxylase; Trx, thioredoxin; Tyr, tyrosine; TyrH, tyrosine hydroxylase

binding affinity to dopamine D2 receptors [55]. Methylated dopamine can reduce the efficacy of AADC-DY treatments targeted to enhance dopamine function.

The 3rd reason for the higher 3-OMD levels may be related to the differential transport of L-dopa and 3-OMD across the blood–brain barrier (BBB). Like in the CSF, the level of 3-OMD in the plasma of AADC-DT subjects is high, approximating 2312% of the normal median range [24]. Both L-dopa and 3-OMD can cross the BBB into the CNS via a facilitated transport mechanism which is shared with other amino acids [56]. This bidirectional transport is unequal between L-dopa and 3-OMD, as 3-OMD is able to compete with the CNS uptake of L-dopa at the BBB [57].

In vivo studies show that a single intracerebroventricular (icv) injection of 3-OMD decreases dopamine turnover rate in the rat striatum and inhibits dopamine transporter and uptake in the brain striatal membrane, and 3-OMD administration for 5 days significantly reduces catecholamine levels and impairs locomotor activities [45]. These adverse effects have been attributed to the 3-OMD ability to exert oxidative stress and cytotoxicity [45]. 3-OMD is also able to enhance the L-dopa prooxidant and cytotoxic effects [45]. Therefore, the high 3-OMD level and accumulation in the AADC-DT brain have the potential to interfere with dopamine synthesis and/or function, and reduce the efficacy of treatment modalities aiming to enhance dopamine production and function. Therefore, 3-OMD has the potential to contribute neuronal injury and death, both directly and through enhancing L-dopa adverse effects, as described in the following paragraphs.

4-Dihydroxy-L-Phenylalanine (L-dopa)

The effects of L-dopa on dopaminergic neurons have been extensively studied in relation to PD. L-dopa, the precursor of dopamine, in combination with carbidopa, which prevents peripheral breakdown of L-dopa by AADC enzyme, is a common treatment modality in PD, aiming to restore brain dopamine levels. However, long-term L-dopa administration and the resulting high brain L-dopa levels were thought to play a role in neuronal injury in PD [58], and concerns have been raised on the contribution of chronic high levels of L-dopa to PD progression [59]. Studies in experimental models of PD report several protein expression profile differences and increased ratio of post-translational protein modifications in the dopamine depleted side of the brain when compared to the corresponding intact side [60]. In addition, proteomic studies in animal model of PD show the sensitivity of dopamine-depleted striatum to the first ever L-dopa exposure, causing irreversible rapid post-translational modification-based changes to several proteins involved in chronic L-dopa adverse effects [61].

L-dopa Autooxidation

L-dopa can exert neurotoxicity via several mechanisms. Undergoing autooxidation, L-dopa is able to generate hydroxyl and superoxide radicals, semiquinones, and quinones [62]. A recent rodent study shows that oral administration of 100 mg/kg L-dopa for 30 days significantly enhanced oxidative stress in rat striatum, indicated by a reduced GSH/GSSG ratio [63]. L-dopa also increased the level of malondialdehyde [63], a marker of lipid peroxidation, and led to a significant increase in glial fibrillary acidic protein (GFAP) immune expression. GFAP, a cytoskeletal intermediate filament, has long been used as an astrocyte-specific marker of cell activation and proliferation. However, astrocyte

proliferation is not a universal response to all damages and can occur in the absence of disease and depends on the context of inflammation and neurodegeneration [64]. Histological examination of the striatal tissues showed L-dopa-induced microglial activation, neurons exhibiting degenerative changes and apoptotic cell death [63]. Similar results were reported in *in vitro* studies showing a relationship between L-dopa-induced oxidative stress and mitochondrial dysfunction, cell apoptosis, and reduction in neuronal cell survival [59, 65–71] in time- and dose-dependent manners [65, 72]. These adverse effects are enhanced in the presence of 3-OMD [45].

Several *in vitro* studies report protective effects of subtoxic concentrations of L-dopa in neurons cocultured in the presence of glial cells [73, 74]. The neuroprotective effects of L-dopa in neurons-glial coculture were thought to be related to the L-dopa's ability to induce the release of GSH, the glial major cellular antioxidant enzyme [74], an effect that seems to be independent of dopamine production [75]. The release of glial GSH is attributed to the ability of a compound to auto-oxidize [69]. As auto oxidizable compounds, subtoxic concentrations of both L-dopa [73] and dopamine [76] are able to induce GSH release from glial cells. However, L-dopa loses this ability in the absence of AADC enzyme [69] and in the presence of high levels of 3-OMD [74] and transition metals [77], suggesting that AADC-DT neurons are highly prone to injury. In young children, the under-developed excretory system can lead to accumulation of the transition metals, especially in under-developed countries due to higher daily exposure [78], a fact that could increase L-dopa neurotoxicity in AADC-DT children living in these regions. Furthermore, dopaminergic neurons and their projection targets are especially vulnerable to L-dopa cytotoxicity, since these neurons are known to possess relatively low glia cells' density [79].

One could argue that the observed cytotoxicity may be attributed to 3-OMD and or dopamine rather than L-dopa. Although 3-OMD does not undergo autooxidation [69], it is still capable of inducing oxidative stress [45]. Similar to L-dopa, dopamine undergoes autooxidation, with the generation of reactive quinones [80]. L-dopa also undergoes enzymatic degradation by MAO with the generation of hydrogen peroxide and hydroxyl radicals [81]. Nevertheless, L-dopa cytotoxicity seems to be dopamine [75], 3-OMD- [67], and MAO- [65] independent, since it is apparent in the presence of the AADC inhibitor, NSD-1015, the COMT inhibitor, Ro 41-0960, and the MAO inhibitor, deprenyl, respectively. Therefore, despite the relatively short biological half-life, the chronic presence of L-dopa in the brain of AADC-DT subjects has the potential for significant neurotoxicity, independent of 3-OMD, but in synergy with it. In addition, L-dopa-induced prooxidant environment is enhanced in the absence of dopamine, as it is the case in AADC-DY, since

dopamine is known to upregulate GSH synthesis, as well as enhance GSH trafficking from glial cells to neurons [82].

Mechanisms Involved in Neuroinflammation

Glial Activation

Chronic oxidative stress brought about by L-dopa and 3-OMD, and by homocysteine (Hcy) (discussed in the following paragraphs), can injure neurons, triggering the recruitment and activation of the microglia, the resident innate immune cells of the brain, which are known to normally regulate several physiological processes required for proper neuronal survival and brain function. Microglial activation has the potential for secreting a wide array of proinflammatory mediators including cytokines, chemokines, reactive oxygen–nitrogen species, and excitotoxins, such as glutamate, resulting in a vicious cycle of neuroinflammation, culminating to neuronal cell death, with dopaminergic neurons to be especially susceptible to neurotoxicity [33, 34]. These pathological processes play major roles in many pathophysiological processes that underly PD [83–85], Alzheimer's disease (AD) [86, 87], and amyloid lateral sclerosis (ALS) [88, 89].

Microglial activation is sustained by a self-feedback loop involving the proinflammatory mediator, the high-mobility group protein box-1 (HMGB1), assisting with the transcription of proinflammatory genes [90]. In addition, there is a crosstalk between microglia and astrocytes, the other type of glial cells. Astrocytes proliferation and activation can be induced through proinflammatory cytokines secreted from the activated microglia [35, 36], further fueling the cascade of neuroinflammation. Therefore, microglia and astrocytes' activation and the intimate crosstalk among the two are fundamental events in neuroinflammation. The damaged neurons can further activate glial cells by releasing ATP and other damage-associated molecular patterns (DAMPs) such as HMGB1 and nucleotides. Interacting with pattern recognition receptors, DAMPS can further perpetuate the cycle of inflammation [91].

Astrocytes are one of the most numerous glial cells in the CNS. Astrocytes' reactivity is highly heterogenous and depends on the type of stimuli with the potential of two distinct functional phenotypes termed A2 as anti-inflammatory and neuroprotective, and A1 as proinflammatory [36, 64]. The A2 can upregulate neurotrophic factors and thrombospondins, able to promote neuronal growth and support synaptic repair [92]. A2 reactive astrocytes also produce anti-oxidative molecules in response to oxidative stress able to protect dopaminergic neurons [93], and have the potential for preventing glutamate excitotoxicity by taking up the glutamate released into the synaptic cleft [94].

IL-1 α and TNF α , secreted from activated microglia, induce the cytotoxic A1 reactive astrocytes' phenotype [35, 36]. A1 astrocytes lose the ability to promote neuronal survival, outgrowth, and synaptogenesis, and upregulate proinflammatory factors, IL-1 α , IL-1 β , and TNF- α [36], leading to the amplification of the microglia-driven inflammatory responses, which when combined with the astrocytes' loss of glutamate-removing activity [95], would promote a neurotoxic environment. The reduced astrocytic glutamate uptake is the result of increased glutamate derived from activated microglia, which downregulates the expression of astrocytic glutamate transporters [96]. Therefore, the increase in inflammatory mediators and glutamate acts in concert to perpetuate neuronal cell death. Furthermore, astrocytes have the capacity to protect neurons against oxidative damage due to the preferential activation in astrocytes of the nuclear factor erythroid-2-related factor 2 (Nrf2), a transcription factor that governs an array of detoxifying and antioxidant enzymes [97]. However, reactive astrocytes may lose the ability to protect neurons due to Nrf2 downregulation [98].

The A1 phenotype is abundant in many neurodegenerative diseases such as PD, AD, and ALS, contributing to death of neurons and oligodendrocytes [36]. The role of astrocytes in the context of neuroinflammation in AADC-DY is specifically important since striatal astrocytes express dopaminergic receptors and D4-mediated signal transduction in response to dopamine [99]. The dysfunction of astrocytes combined with the absence of dopamine neuroprotective effects [82, 100] would have deleterious effects on dopaminergic neurons in AADC-DY. In addition, striatal astrocytes act as a reservoir, taking up the excess L-dopa [101]. However, the activated astrocytes may have an impaired ability to take up the excess L-dopa, similar to the loss of glutamate-removing activity [95].

Mitochondrial Dysfunction

As dynamic organelles, mitochondria adapt to physiological or environmental changes in cellular requirements, by processes of transport, biogenesis, fusion, fission, and mitophagy, the latter refers to the removal of damaged mitochondria through autophagy, which involves the two proteins, Parkin and Pink1 [102]. The reciprocal interactions between these processes are integral to mitochondrial homeostasis [103], and the increase in oxidative stress is able to adversely affect the different functions of mitochondria [37].

In microglia, oxidative stress-induced microglial activation leads to mitochondrial dysfunction. Fragmented mitochondria released from microglia are able to not only trigger A1-inflammatory-astrocytes' activation [104], but also exacerbate the pro-inflammatory microglial M1 phenotype [105] and the release of neurotoxic cytokines, further enhancing the ROS formation [106].

Mitochondria are essential for ATP production through oxidative phosphorylation, involving a subset of the mitochondrial DNA (mtDNA). Compared to other cell types, neurons are more dependent on mitochondrial oxidative phosphorylation to fulfill their energy demands. This process generates reactive oxygen species (ROS). Neurons have limited capacity to upregulate glycolysis or to counteract oxidative damage. The generated ROS has the potential to damage mtDNA [107], resulting in mitochondrial dysfunction and dopaminergic neuronal cell loss [108]. A correlation between altered mitochondrial dynamics and neurodegeneration has been observed in PD and AD [109].

Functional astrocytes have the ability for reducing neuronal-derived ROS via enhancing the production of antioxidant molecules [93], as well as internalizing and degrading dysfunctional mitochondria [110], in order to protect neurons and maintain the quality of mitochondria for bioenergetic functions. However, reactive astrocytes may lose the capacity to reduce oxidative stress due to Nrf2 downregulation [98], and have impaired mitophagy capacity due to inflammation-induced Parkin downregulation [111].

Neuroinflammation in AADC-DY

Although the above pathological processes have not been examined in relation to AADC-DY, the neuronal injury and death may contribute to the neuroimaging abnormalities observed in AADC-DT subjects, such as cerebral [4] and cortical [112] atrophy, demyelination [113], and degenerative changes of white matter [4]. Furthermore, brain magnetic resonance imaging of 12 AADC-DT subjects showed hypomyelinations, reduced volume of caudate nucleus, and decreased density of the white matter fiber tracts [114]. Oxidative stress and microglial activation are known to also adversely affect BBB integrity [115, 116], especially in the presence of high Hcy levels [117].

Inflammation-induced loss of astrocytes' neuroprotective functions can also adversely affect the BBB integrity, since astrocytes regulate brain microvascular permeability via astrocyte-endothelial communication and the release of regulatory factors, such as transforming growth factor (TGF)- α and glial-derived neurotrophic factor (GDNF); these factors are able to influence the permeability of tight junction in endothelial cells [118]. The increase in BBB permeability can result in the infiltration of systemic inflammatory mediators and immune cells into the CNS, further promoting microglial activation and neuronal cell death. The increase in striatal BBB permeability has been thought to underly PD disease progression [119], and could similarly be involved in the pathophysiology of AADC-DY.

It is noteworthy that the AAV2 vector used for the transfer of the functional hDDC gene has high degree of tropism to neurons, and low degree of tropism to

microglia and astrocytes [120]. It remains an open question to whether the introduction of a functional copy of the hDDC gene to putamen and/or midbrain of AADC-DT subjects would influence the activation state of glial cells and hence the cascade of neuroinflammatory processes.

Additional L-dopa Neurotoxic Effects

L-dopa-Induced Excitotoxicity

L-dopa can also induce glutamate excitotoxicity in a Ca(2+)-dependent, autooxidation-independent manner, although the timeline for neuronal injury and death due to L-dopa-induced glutamate excitotoxicity is delayed when compared to the timeline of neuronal injury and death due to L-dopa autooxidation [100]. Studies show that ischemia-induced elevation of endogenous L-dopa increases glutamate release in rat striatal neurons, an effect that is Ca(2+) dependent and dopamine independent [100], suggesting that elevation of endogenous L-dopa has the potential to exert neurotoxicity similar to the elevation due to L-dopa administration [121, 122]. The Ca(2+)-induced enhanced glutamate release and the activation of the postsynaptic *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors [123, 124] lead to caspase-3-activation [125] and neuronal cell death, a common pathway involved in the pathophysiology of many neurodegenerative disorders. Therefore, both L-dopa autooxidation and L-dopa excitotoxicity may work in concert to contribute to neurodegeneration.

The L-dopa-induced glutamate-related excitotoxicity inhibits the AADC enzyme both in the striatum and in substantia nigra (SN) [41], and therefore, can adversely affect the clinical outcomes of AAV2-mediated hDDC gene therapy in AADC-DY. This notion is further supported by the results of a study showing that the central inhibition of the AADC enzyme by NSD-1015 amplifies the endogenous L-dopa-induced glutamate release and striatal neuronal cell death [100]. This is because dopamine negatively controls glutamate release and therefore dopamine depletion would have the potential to enhance glutamate release [100, 126]. The results collectively suggest that the L-dopa-induced glutamate excitotoxicity (as well the Hcy-induced glutamate excitotoxicity described in the following paragraphs) is likely to be potentiated, playing a role in dopaminergic neuronal injury and death in AADC-DY.

L-dopa-Induced Protein Misfolding

An additional mechanism contributing to L-dopa cytotoxicity stems from structural similarities between L-dopa and the amino acid tyrosine. Due to these structural similarities,

L-dopa can replace tyrosine in the polypeptide chain of proteins in the brain, promoting protein misfolding and protein aggregation with an adverse effect on mitochondrial function [127]. The glial dysfunction and the impaired mitophagy [111] can lead to the accumulation of misfolded proteins, enhancing neurodegeneration. The aggregation of misfolded brain proteins is the underlying cause of neuronal damage in several neurodegenerative disorders [128], and likely contribute to neurodegeneration associated with AADC-DY.

L-dopa-Induced Serotonergic Dysfunction

The dense serotonergic fibers projecting to the striatum are capable of a high affinity L-dopa uptake and conversion to dopamine [129]. Because of the dopaminergic lesions in PD, the serotonergic neurons are the main cells to use the exogenous L-dopa for dopamine synthesis in the striatum and SN [130]. Studies in a rodent model of PD show that high levels of brain L-dopa can also adversely affect the serotonergic neurons, with the reductions in both serotonin and dopamine in different parts of the brain, including striatum and SN [131], resulting in serotonin and 5-HIAA depletion and behavioral and cognitive dysfunctions [132]. Similarly, the chronic administration of L-dopa has been thought to exert toxicity toward serotonergic neurons, hence contributing to physiological dysfunction in PD patients [133].

It is unknown whether the high levels of endogenous L-dopa found in AADC-DY undergo uptake into serotonergic neurons like the exogenous administered L-dopa does, but if that is the case, then it can exert adverse effects on serotonergic neurons like it does in dopaminergic neurons, influencing their long-term survival. L-dopa adverse effects in serotonergic neurons would be enhanced due to serotonin depletion in AADC-DY since serotonin is known to exert antioxidant and neuroprotective effects [134, 135]. Furthermore, striatal astrocytes express serotonergic receptor 5-HT_{1A}, a key mediator of serotonergic signaling in the CNS, and stimulation of these receptors activates Nrf2 in astrocytes, with the end results of upregulating the astrocytic antioxidant and neuroprotective capacities [136]. However, in AADC-DY, this neuroprotective pathway may be impaired due to glial dysfunction and serotonin deficit, negatively impacting neuronal survival.

L-dopa-Upregulation of Kynurenine Pathway

L-dopa oxidation has the potential to also upregulate the kynurenine pathway (KP), the major pathway in tryptophan catabolism, culminating in the generation of metabolites such as quinolinic acid [38, 39], known to exert neurotoxic effects via both increase in glutamate release and stimulation of NMDA receptors [40], further fueling oxidative stress and mitochondrial dysfunction [137]. Although high 5-HTP may

contrast some of the L-dopa prooxidant effects [138], 5-HTP is known to dose-dependently inhibit AADC enzyme activity [139], and therefore could interfere with treatments aiming to increase dopamine and serotonin productions.

Quinones, the byproducts of L-dopa oxidation, can inactivate tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, adversely influencing serotonin production. The quinones exert these adverse effects through both modification of the cysteinyl residues within tryptophan hydroxylase, and via converting tryptophan hydroxylase to a redox-cycling quinoprotein; the latter is able to fulfill the role of a radical with neurotoxic properties [140]. Furthermore, quinones can covalently bind Parkin, inactivating ubiquitin ligase function of Parkin, thus impairing mitophagy in dopaminergic neurons [141], suggesting the vulnerability of these neurons to quinone toxicity. The data cumulatively demonstrate the many pathways through which the excess L-dopa is able to exert cytotoxicity, thus promoting neuronal injury and death in AADC-DY.

Homocysteine Neurotoxicity

Homocysteine (Hcy) is a non-protein, sulfur-containing amino acid that is generated during methionine transmethylation via several steps catalyzed sequentially by SAM synthetase, methyltransferase (MT), and SAH hydrolase enzymes [142]. Higher levels of Hcy have been regarded to be involved in the pathology of several disorders including neurodegeneration [143, 144]. PD patients show a positive correlation between Hcy plasma levels and L-dopa [145] and 3-OMD [146] concentrations. The effect of L-dopa on Hcy plasma levels in PD subjects is more pronounced under folate deficiency [147]. Neither plasma nor CSF levels of Hcy have been measured in AADC-DY. However, the excess brain L-dopa availability in AADC-DY, and the increase in the synthesis of 3-OMD by COMT enzyme, would result in the accelerated production of SAH, which would be readily converted to Hcy and adenosine by the action of SAH hydrolase enzyme, in a folate-dependent reaction, culminating to a reduced cerebral methyl donor, SAM, and reduced folate as the co-factor [148].

Homocysteine-Induced Oxidative Stress

Several mechanisms account for the adverse effects of Hcy. The subcutaneous administration of Hcy to rats increased both the plasma and brain levels of Hcy [149]. In the brain, Hcy exerted significant oxidative stress [149] and increased the level of inflammatory mediators resulting in mitochondrial dysfunction, DNA damage, and neuronal cell death, as measured in the hippocampus/cerebral cortex [150]. Similar adverse effects were observed after intracerebral injection of Hcy in mice, showing

significant increase in lipid peroxidation and neuroinflammation which were accompanied with reduced GSH levels, culminating to cortical damage and cognitive deficit [151].

The oxidative stress, inflammation, and mitochondrial dysfunction in striatum and cerebellum have been also shown under chronic mild hyperhomocysteinemia [152], accompanied by a dose-dependent loss of striatal dopaminergic neurons, and motor dysfunction [153]. In PD subjects, Hcy plasma levels correlate with cognitive dysfunction [154] and Hcy-induced loss of dopaminergic neurons is thought to contribute to PD neurodegeneration [153].

It is assumed that Hcy promotes oxidative stress via reactive oxygen species generation upon disulfide bond formation. However, additional studies show that Hcy, unlike other thiol compounds, possess distinctive features enabling it to easily forms disulfide bonds with free thiol groups of cysteine residues in proteins forming thermodynamically stable Hcy-thyl-cysteine able to act as a radical after undergoing hydrogen atom transfer reaction [28]. This reaction can lead to the oxidation of DNA and proteins and limit the availability of the antioxidant precursor, cysteine.

Hcy reduces catecholamine and serotonin levels in the brain and increases MAOB activity [29]. The increase in MAOB will lead to accelerated dopamine catabolism to 3,4-dihydroxyphenylacetaldehyde (DOPAL), a reactive aldehyde derivative with cytotoxic effects [155]. The increase in MAOB can also reduce the activity of the antioxidant enzyme, superoxide dismutase (SOD) [156]. Furthermore, the BBB is especially sensitive to Hcy and even a mild increase in Hcy level can compromise BBB integrity, independent of Hcy-induced neuroinflammation [157]. Hcy neuronal damage is likely to occur in AADC-DY because of chronic presence and accumulation due a relatively long biological half-life (4 h) [158], with the potential for contributing to both motor and cognitive dysfunctions [29], the severity of which would be the function of both Hcy concentration and the length of exposure of neurons, especially dopaminergic neurons, to the insult.

Homocysteine-Induced Excitotoxicity

Similar to L-dopa, Hcy can exert excitotoxicity by interaction and activation of the NMDA receptors [159, 160], an adverse effect that is potentiated in the presence of high levels of glycine [159]. Glycine is known to be a NMDA receptor agonist [161]. AADC-DT subjects present with high CSF levels of glycine [8] with the potential to synergize with Hcy, further augmenting excitotoxicity and subsequent neuronal injury and death. Hcy activation of NMDA receptors is associated with the induction of seizures in young rats [162]. Whether cases of single seizures observed in some AADC-DT subjects [13] are related to Hcy excitotoxicity warrants further investigations.

Dopaminergic neurons extend their axons to the striatum, releasing dopamine, and striatal astrocytes express dopamine receptors, including D2 receptors [99]. A2 adenosine receptors and D2 dopaminergic receptors closely interact in an antagonistic manner to modulate glutamatergic transmission in striatal astrocytes through receptor heteromerization; D2 receptors' activation inhibits glutamate release, while activation of A2A receptors abolishes the effect of D2 receptor-mediated inhibition of glutamate release from astrocytes [163]. Therefore, the intercellular communication between neurons and astrocytes via A2A-D2 heteromer can contribute to glutamatergic regulation.

Hcy has been shown to inhibit D2-mediated inhibition of glutamate release without affecting the functional interaction between A2A receptors and D2 receptors [164]. The absence of inhibition of glutamate release, due to dopamine depletion in AADC-DY, and the presence of high levels of Hcy and adenosine are factors leading to enhanced Hcy-induced excitotoxicity with adverse effects on dopaminergic neurons. Furthermore, similar to Hcy, high level of adenosine co-released with Hcy is able to increase SAH [165] and therefore can indirectly downregulate transmethylation processes. Such adenosine adverse effect is likely to be accentuated in the presence of high Hcy levels. Adenosine activation of A2 adenosine receptors is known to contribute to dopaminergic neurodegeneration, and antagonists to this receptor are considered for their beneficial effects in the treatment of PD [166]. The results collectively demonstrate the deleterious effects of chronic brain exposure to Hcy, a scenario likely to exist in AADC-DY.

Reduced Homocysteine Metabolism

Hcy levels are determined by the balance between biosynthesis and catabolism; the higher expected Hcy levels in AADC-DY can be the result of not only increase in the synthesis, but also reduced Hcy catabolism. Hcy is catabolized by two means: remethylation to methionine and transsulfuration to cysteine. Hcy remethylation occurs in both folate/vitamin B12-dependent and vitamin B12-independent mechanisms. The B12-dependent uses N-5-methyl tetrahydrofolate (MTHF) as methyl group donor catalyzed by methionine synthase (MS) with the aid of vitamin B12 as cofactor, whereas the remethylation pathway is independent of vitamin B12 and relies on betaine as methyl donor catalyzed by betaine-homocysteine methyltransferase (BHMT) enzyme.

Hcy transsulfuration is catalyzed by the two vitamin B6-dependent enzymes, the cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE), as well a third enzyme, 3-mercaptopyruvate sulfurtransferase (MST). CBS is expressed mainly in glial/astrocyte lineage [167], whereas CSE [168] and 3-MST [169] catalyze Hcy metabolism

mainly in neurons. The first step is the condensation between Hcy and serine by CBS produces cystathionine, which is further hydrolyzed by the CSE enzyme to produce cysteine and α -ketobutyrate [142, 170]. Cysteine is a precursor, critical for the synthesis of the antioxidant GSH and for the synthesis of H₂S, a signaling molecule able to exert neuroprotective effects via multiple biological mechanisms including antioxidative, anti-inflammatory, and antiapoptotic [171].

The high Hcy levels, brought about by the accelerate rate of L-dopa-induced *o*-methylation, are able to diminish CBS enzyme activity [29] as well as reduce the level of the methyl donor, SAM, known to be the allosteric activator of the CBS enzyme [30], hence resulting in further Hcy accumulation. Hcy catabolism is also hampered due to dopamine and serotonin depletion since both neurotransmitters are known to upregulate CBS expression [172]. Therefore, factors such as depleted neurotransmitters, reduced SAM [8], high Hcy and adenosine levels, and depletion of cerebral folate due to L-dopa-induced oxidative stress [173] all have the potential for interfering with CBS enzyme activity and remethylation cycle, thus impairing Hcy catabolism in AADC-DY. This scenario is especially relevant to the brain due to the absence of BHMT enzyme [174], and brain dependency on folate/vitamin B12-pathway for Hcy remethylation, rendering the brain vulnerable to increased Hcy levels.

The physiological levels of CSE enzyme are especially low in the brain; in fact, CSE levels in the brain are > 100 folds lower than that in the liver [175] and more than 40-fold higher cystathionine levels are reported in the brain compared to other human tissues [176]. Low physiological levels of CSE enzyme in the brain combined with oxidative stress induced by L-dopa, 3-OMD [177], and Hcy [29] could significantly reduce CSE activity, resulting in Hcy accumulation, and subsequent reduction in the production of cysteine and its neuroprotective metabolites, GSH and H₂S.

Homocysteine Downregulation of Cellular Antioxidant Status

AADC-DY-related deficits in dopamine and serotonin and the reduced activities of CBS and CSE enzymes [172], combined with reduced level of SAM [178], all are factors that could adversely affect H₂S production and therefore, the antioxidant capacity of the brain, an organ known to be especially sensitive to oxidative stress [179]. CBS and CSE enzymes can both produce H₂S directly from cysteine or through catalysis of the condensation of cysteine + cysteine and Hcy + cysteine. In addition, CSE can produce H₂S directly from Hcy or through catalysis of the condensation of Hcy + Hcy [180]. The reactions catalyzed by both CBS and CSE are all vitamin B6-dependent [180]. In normal physiological state, CBS is the major enzyme in the production of H₂S [181], which

takes place mainly in astrocytes [182]. However, in the presence of high brain Hcy, as it is likely to be the case in AADC-DY, CSE becomes the principal enzyme for the H₂S generation through CSE catalysis of Hcy + Hcy condensation [183]. Therefore, the physiologically low brain CSE levels and the reduced CSE activity in AADC-DY would interfere with H₂S formation, promoting an environment of low antioxidant capacity, where the cytotoxicity of L-dopa is potentiated [68]. This prooxidant environment is further enhanced by the ability of Hcy to interact with H₂S, forming Hcy-persulfide, thus reducing H₂S bioavailability [184]. The disturbances in the H₂S levels have been shown in many neurodegenerative diseases including PD and AD [185], and likely to be a part of AADC-DY pathophysiology.

H₂S in the brain can be also generated by MST enzyme using 3-mercaptopyruvate (3-MP) as substrate, with thioredoxin (Trx) and dihydrolipoic acid (DHLA), the reduced form of α -lipoic acid (ALA) as cofactors [186]. Both Trx and DHLA act as sulfur acceptors for MST-bound cysteine persulfide (an intermediate generated by sulfur transfer from 3-MP to cysteine catalyzed by MST) to generate H₂S [168, 187]. 3-MP is produced by the action of cysteine aminotransferase (CAT) enzyme on cysteine in the presence of α -ketoglutarate [180]. However, the physiological level of MST enzyme in the brain is 10 folds lower than the levels in the liver [188], and the oxidative stress exerted by the different metabolites could further reduce MST activity [31]. The chronic high level of L-dopa can also interfere with H₂S production via reducing the level and the activity of the cofactor Trx in neurons [32]. Since H₂S negatively regulate Hcy levels [189], the reduced production of H₂S leads to further Hcy accumulation in the brain.

One of the many H₂S biological effects in the brain is the regulation of the gastric juice, by reducing the gastric PH [190]. Most of AADC-DT subjects suffer from gastrointestinal problems, including digestion, diarrhea, constipation, reflux, nausea, and vomiting [191]. These clinical symptoms may be in part the result of hypochlorhydria due to reduced brain H₂S production. Furthermore, H₂S has been shown to regulate glucose homeostasis in a number of organs including hepatocytes, skeletal muscles, and the brain [192]. It is well known that neurotransmitters such as dopamine and serotonin regulate blood glucose [193]. The hypoglycemia observed in AADC-DT subjects may be the result of both neurotransmitters' deficiency and H₂S dysregulation.

Homocysteine Reduction in Cellular Methylation Status

Another consequence of elevated Hcy levels is the accumulation of SAH, since as a result of inefficient removal

of Hcy through remethylation or degradation to cysteine, the equilibrium constant of SAH hydrolase favors SAH synthesis rather than hydrolysis (reverse catalysis) [175]. SAH elevation is known to disturb cell methylation capacity through the ability to competitively bind the catalytic region of most SAM-dependent methyltransferases, inhibiting transmethylation reactions [194], including the formation of SAM. The results of a combined genetic and dietary approach in rats shows an inverse relationship between SAM and SAH and a positive relationship between plasma Hcy and brain SAH [195]. The increase in SAH seems to be a better indicator of cellular methylation capacity than decrease in SAM [195]. Therefore, high levels of Hcy and SAH may play a role in reduced SAM levels observed in AADC-DY [148], suggesting suboptimal cellular antioxidant and methylation capacities.

Brain may be especially sensitive to SAH adverse effects since brain has a relatively low SAH hydrolase activity [175], and BHMT, the alternate Hcy remethylating enzyme, is not active in the brain [174]. Hypomethylation can adversely affect DNA and proteins, with adverse effects including neuronal susceptibility to injury and apoptosis [196]. SAM/SAH ratio also correlates with total GSH level and GSH/GSSG ratio [53]. The SAM/SAH ratio in plasma of healthy individuals is calculated to be 4.9 ± 1.7 (range, 1.6–9.5) [197], but this ratio is reduced in PD subjects, correlating with cognitive dysfunction [54]. Although this ratio is unknown, it is expected to be reduced in AADC-DT subjects as a result of L-dopa-induced reduction in SAM [198] and Hcy-induced increase in SAH.

Homocysteine Oxidation-HCA Generation

In a prooxidant environment, Hcy is prone to undergo oxidation generating 2-amino-4-sulfo-butanoic acid (homocyst(eic acid, HCA) [27], with enhanced production under folate deficiency [199]. HCA is an analogue of glutamic acid, able to bind and activate NMDA receptors. Even very low concentration of HCA is capable of over-activating the NMDA receptors leading to very high brain toxicity [200]. In fact, HCA is significantly more neurotoxic than Hcy [201, 202]. The icv infusion of HCA to immature rats caused seizures recorded in various parts of the brain, including striatum [203]. Brain levels of HCA are increased in the rodent model of AD, triggering memory deficit [204], and the administration of an anti-HCA antibody prevents cognitive impairment [204]. Compounds that reduce HCA to Hcy, such as H₂S, have been shown to improve cognitive function in AD patients [205]. Similarly, ferulic acid, which inhibits HCA binding to NMDA receptors, improves HCA-induced cognitive impairment [205]. The data demonstrates the detrimental effects of HCA in the brain in the presence of high Hcy and a prooxidant environment that characterizes AADC-DY.

We propose that in the absence of AADC enzyme [69], and the absence of dopamine [82, 100] and serotonin neuroprotective effects [134, 135], the chronic presence of high levels of L-dopa, 3-OMD, and Hcy, would act in concert and synergy to generate a prooxidative and excitotoxic environment, resulting in glial activation, mitochondrial dysfunction, and neuroinflammation, hence marking the onset of neurodegeneration in AADC-DY. This hostile environment faced with the metabolites-induced suboptimal cellular antioxidant capacity would negatively impact neuronal survival in general and dopaminergic neurons in particular. The extent and severity of adverse effects would be in turn the function of the levels and duration of exposure to the toxic metabolites. This conclusion is supported by the results of the clinical outcomes of AAV2-mediated hDDC gene therapy to be more favorable in younger AADC-DT patients compared to older ones [18, 206], suggesting that the relatively shorter duration of the disease in younger subjects may be associated with a less opportunity for the toxic metabolites to cause an irreversible neuronal injury, compromising neurons' functional integrity.

AADC-DY Novel Treatment Strategies

We have extensively discussed several upregulated metabolites in AADC-DY and their synergistic role in inducing oxidative stress and neuroinflammation, as well as interfering with the synthesis of cellular antioxidants. These effects of the metabolites combined with the absence of dopamine and serotonin antioxidant and neuroprotective effects would generate an unreceptive milieu impeding the success of AADC-DY treatments aiming to enhance neurotransmitters' synthesis and or function. To improve treatment efficacy, either the levels of metabolites should be reduced or alternatively the cellular antioxidant capacity be increased by either supplementing with compounds that exert antioxidant effects or with precursors involved directly or indirectly in the generation of cellular antioxidants. These interventions, singly or combined, would be more effective if applied early in the course of the disease, before irreversible neuronal injury occurs. These novel strategies are depicted in Fig. 2.

COMT Inhibitors

Among strategies for reducing the levels of metabolites, and restoring the cellular antioxidant and methylation capacities is the use of COMT inhibitors. Although not a part of treatment modality in AADC-DY, treatment of PD patients with the COMT inhibitor, tolcapone, results in a marked reduction in 3-OMD plasma level [207]. COMT inhibitors can also contrast the increase in Hcy levels, minimizing the related toxicity brought about L-dopa-induced accelerated

o-methylation [208]. In addition, tea polyphenol, especially epigallocatechin-3-gallate (EGCG), and (+)-catechin are effective COMT inhibitors, known to reduce 3-OMD levels in rat striatum [209, 210]. Having adequate bioavailability, these compounds cross BBB, exerting antioxidant, anti-inflammatory, and neuroprotective effects, even in low concentrations [209, 210]. The data suggests the possible benefits of treatment of AADC-DY with centrally acting COMT inhibitors as a mean for reducing 3-OMD and Hcy levels and improving both SAM/SAH and GSH/GSSG ratios.

H2S Donors

Alternatively, one can enhance the cellular antioxidant capacity to counteract metabolites-induced oxidative stress. Rodent studies show that intraperitoneal administration of the H2S donor, sodium hydrogen sulfide (NaHS), alleviated Hcy-induced toxicity by reducing oxidative damage, neuroinflammation, cells apoptosis, neurodegeneration, and cognitive deficit [29, 149, 151]. H2S administration also lowered the plasma and brain levels of Hcy, increased the level of the endogenous H2S, restored the activities of CBS and CSE enzymes, increased catecholamine levels, and improved cognitive and behavioral deficits in Hcy-treated animals [29, 151]. NaHS treatment of a 6-OHDA-induced PD model attenuated neuronal loss, lipid oxidation, and the accumulation of inflammatory markers, and improved movement dysfunction [229]. Similar effects have been shown in the MPTP-induced PD model, where H2S inhalation prevented movement disorder and the degeneration and apoptosis of TH-containing neurons [230]. In addition, the inhalation of low H2S levels has been shown to increase plasma glucose levels in postpartum rats [231], suggesting that such strategy may not only impact neurodegenerative processes, but also improve the hypoglycemia often reported in AADC-DT subjects.

Having a relatively high expression in the brain, the neuroprotective effects of H2S is attributed to the ability to prevent oxidative stress through Nrf2 upregulation [232] and preserve mitochondrial function, through upregulating Parkin activity [233]. H2S donors have been suggested as treatment modality for PD [234, 235], where the loss of Parkin activity has been documented [236]. These studies collectively suggest the beneficial effects of H2S as a part of AADC-DY treatment modality to enhance cellular antioxidant status, prevent mitochondrial dysfunction, and minimize neuronal injury.

Sulfur-Containing Compounds

The increase in H2S production can also be achieved by administration of several sulfur-containing compounds including SAM, methionine, N-acetylcysteine (NAC),

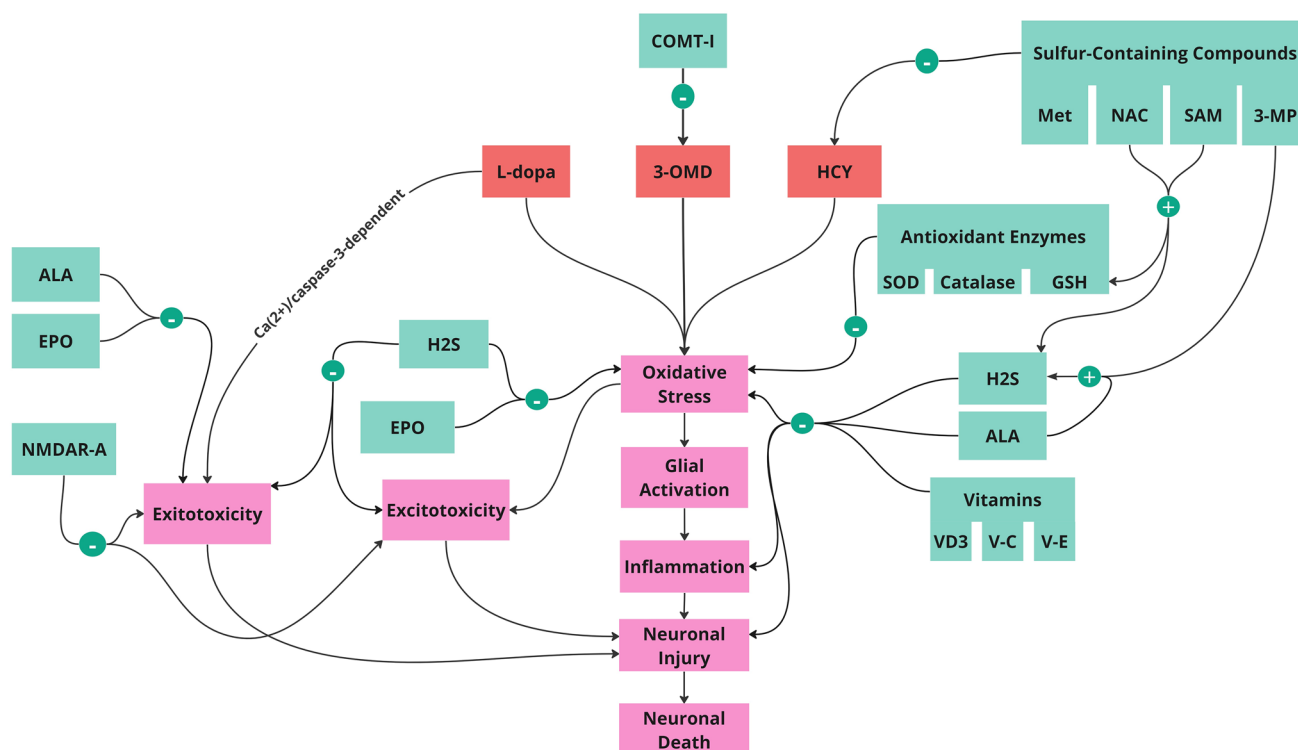


Fig. 2 Strategies to improve the treatment of AADC-DY. The new concept of AADC-DY as a neurodegenerative disease offers additional strategies to treat the disease. In addition to COMT inhibitors [207–210], H₂S donors [29, 149, 151], sulfur-containing compounds (SAM, methionine, N-acetylcysteine, and 3-MP) [211–213], and the powerful antioxidant ALA [214–216] are strategies that could restore cellular methylation and cellular antioxidant capacities. Other strategies include the use of antioxidant enzymes (GSH, SOD, catalase) [217], vitamins such as ascorbate [132, 218, 219], α -tocopherol [45, 220, 221], and VD₃ [222–225]. NMDA receptor antagonists are able to reduce glutamate excitotoxicity [41, 100], and erythropoietin is able to exert neuroprotective effects [226–228]. These strategies used singly or combined early in the course of the disease will mini-

mize neuronal injury, preserving the functional integrity of the dopaminergic and serotonergic neurons. The red highlights present the upregulated metabolites. The purple highlights present the pathways to neuronal injury and death. The green highlights present the various treatment strategies. Abbreviations: ALA, α -lipoic acid; COMTI, catechol-*O*-methyl-transferase inhibitor; EPO, erythropoietin; GSH, glutathione; Hcy, homocysteine; H₂S, hydrogen sulfide; L-dopa, 3,4-dihydroxy-L-phenylalanine; Met, methionine; 3-MP, 3-mercaptopyruvate; NAC, N-acetyl-cysteine; NMDAR-A, *N*-methyl-D-aspartate receptor-antagonist; 3-OMD, 3-*O*-methyl-dopa; SAM, *s*-adenosylmethionine; SOD, superoxide dismutase; VD₃, vitamin D₃; V-C, ascorbate; V-E, α -tocopherol

and 3-MP. The administration of SAM, an activator of CBS, has the potential for restoring cellular methylation status, lowering Hcy levels, improving SAM/SAH ratio, and enhancing H₂S production [211]. Although the direct effects of SAM supplementation on the brain are not well known, SAM is able to cross the BBB with slow accumulation in the CSF [237]. SAM administration to PD patients is well tolerated reducing the severity of depression [238]. Similarly, supplementation with methionine, the precursor of SAM, can improve the hypomethylation status, reducing Hcy and SAH levels [212], hence leading to increased H₂S production. Supplementation with NAC can increase the levels of cysteine, the precursor for GSH, and enhance H₂S production, while reducing Hcy via Hcy + cysteine condensation [212]. In addition, rodent studies show the potential of 3-MP to increase H₂S; this

effect is enhanced when 3-MP is administered in combination with α -lipoic acid (ALA) [213].

The enhancement in cellular antioxidant capacity can be also achieved by supplementation with antioxidant enzymes including SOD, catalase, and GSH [217], all of which fulfill important roles as antioxidants in the brain. PD patients, at advanced disease stages, show significantly lower SOD level in whole blood and in red blood cells, which correlates with disease duration [239]. Similarly, GSH level in the brain of PD patients is significantly reduced [240]. Studies show that SOD/catalase mimetics exert neuroprotective effects in a herbicide-mediated PD model [241]. Accumulated evidence supports the supplementation and enhancement of GSH and related antioxidants for improving PD pathology [242].

The ability of bromocriptine and pergolide to improve AADC-DY clinical symptoms [8] may stem in part from

upregulating SOD [243]. In addition, studies in animal and cellular models of PD report that MAO inhibitors exert neuroprotective effects in part through increasing SOD and catalase activities in the brain regions containing dopaminergic neurons [244]. Furthermore, the clinical efficacy of vitamin B6 in AADC-DY treatment is attributed in part from B6 ability to induce GSH via Nrf2 activation [245]. The clinical efficacies of the dopamine-receptor agonists, MAO inhibitors, and vitamin B6 and their potential for increasing antioxidant levels, support the use of antioxidant enzymes and their mimetics for improving AADC-DY neuropathology.

Another powerful antioxidant, the organosulfur compound ALA, is an essential cofactor for several enzymes involved in mitochondrial energy metabolism. In vitro studies show the potential of ALA in protecting primary hippocampal and cortical neurons from HCA-mediated toxicity-induced neuronal cell death [214]. In addition, ALA reduced microglial activation and neuroinflammation in the MPTP-induced PD model [246]. In the 6-OHDA-lesioned rats, ALA reduced MDA levels and upregulated GSH activity in the striatum, thus preventing L-dopa activation of the proapoptotic caspase-3 signal in SN, resulting in improved neuronal survival [215]. The neuroprotective effects of ALA are attributed in part to the ability to downregulate inflammatory processes through targeting the proinflammatory transcription factor, the nuclear factor- κ B pathway [216].

ALA is synthesized *de novo* in most cells in small amounts, using several intermediates including SAM [247] and therefore, reduced SAM is one of the many factors that may contribute to reduced levels of ALA in AADC-DY. In vivo, ALA could be reduced to its dithiol form, dihydrolipoic acid (DHLA), and in this form it functions as a sulfur acceptor during H₂S production catalyzed by MST enzyme. Upon completion of this reaction, ALA is concomitantly released with H₂S [187]. Therefore, the reduced MST activity and Trx levels are additional factors impeding not only the H₂S production, but also that of ALA in AADC-DY. ALA is available as dietary supplement, and it is used as pharmaceutical drug in some European countries. Use of ALA in AADC-DT subjects, early in the course of the disease, will exert antioxidant and neuroprotective effects, preserving dopaminergic and serotonergic functions.

Vitamins

Metabolite-induced oxidative stress not only could deplete the level of cofactors such as folate [173], but it can also increase the rate of utilization of the two other antioxidant vitamins, ascorbate and α -tocopherol [248], thus adversely influencing cellular antioxidant capacity. In vitro studies show that ascorbic acid blocks L-dopa-induced reduction in the activity of the antioxidant enzyme catalase in mesencephalic cells [249], and prevents L-dopa-induced cell toxicity

in striatal neurons [218]. These results were further corroborated in *in vivo* studies showing that ascorbate administration reduced Hcy, increased the activity of catalase and SOD enzymes in the hippocampus [219], and prevented serotonin depletion in the dorsal raphe nucleus, thus minimizing L-dopa-induced cognitive dysfunction in rats [132].

Similar results have been reported with α -tocopherol, able to reduce L-dopa- and 3-OMD-induced oxidative stress, preventing neuronal injury and motor dysfunction in rats [45]. The natural form of α -tocopherol, namely α -tocotrienol is multifold more potent than α -tocopherol, able to block neuronal injury resulting from not only oxidative stress, but also from Ca(2+)-dependent glutamate-induced excitotoxicity [220]. This potency has been attributed to a faster cellular uptake of the former compared to the latter [250]. Treatment of PD patients with the combination of ascorbate and α -tocopherol reduced progression of the disease, indicated by the extension of the time to L-dopa treatment [221]. In addition, a randomized clinical study compared the benefit of α -tocopherol and NMDA receptor antagonist, memantine, in reducing the functional decline in patients with mild cognitive dysfunction, reporting encouraging results [251].

The other vitamin with the potential benefit in the treatment of AADC-DY is vitamin D3 (VD3), with the active metabolite being 1,25-dihydroxyvitamin D3. VD3 is a hormone with pleiotropic effects; in addition to the classical role in the regulation of intestinal, bone, and kidney calcium and phosphorus absorption, VD3 exerts antioxidant, anti-inflammatory, and neuroprotective effects [222]. Although there are no data on serum level of VD3 in AADC-DT subjects, PD patients show reduced level of this vitamin, and an inverse relationship between VD3 serum level and disease severity [252]. VD3 crosses the BBB [253], able to influence dopaminergic neurons through binding to and activation of the VD3 receptor/retinoic X receptor heterodimeric complex [254]. VD3 receptors act as a ligand-inducible transcription factors. VD3 belongs to the nuclear hormone receptor superfamily [255] with the highest concentrations reported to be located in the SN [256], indicating the important function of these receptors in dopaminergic neurons.

VD3 treatment protected rats' nigrostriatal dopaminergic neurons against a partial lesion induced by the unilateral striatal injection of 6-OHDA, administered either before or after lesion induction [223]. The treatment prevented lipid peroxidation and the increase in the level of proinflammatory mediator TNF- α . VD3 also reversed the 6-OHDA-induced reduction in the level of dopamine transporter, an indicator of dopaminergic membrane integrity, and the level of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, hence protecting dopaminergic neurons [223]. The neuroprotective effects are attributed in part to VD3's ability to increase GDNF level in the striatum and SN, inhibiting cell apoptosis [224]. The results from rodents' studies agree with those of a clinical

trial showing that VD3 supplementation of PD patients prevented disease deterioration measured by modified Hoehn and Yahr stage and Unified Parkinson's Disease Rating Scale [225]. The accumulated evidence support VD3 potential to downregulate pathophysiological processes that characterize neurodegeneration, and therefore may be of benefit in the treatment of AADC-DY.

NMDA Receptor Antagonists

The increase in the brain levels of metabolites also leads to an enhanced glutamate release via both oxidative- and Ca(2+)-dependent pathways as described above. Rodent studies show that L-dopa-induced glutamate release and cell death are prevented by the administration of NMDA receptor antagonists CGP 40116, glycine/NMDA receptor antagonist HA-966, memantine, and the antiparkinsonian drugs bupropion and amantadine, with a parallel increase in the level of the AADC enzyme [41]. Similar results were observed with the competitive DOPA antagonist, DOPA cyclohexyl ester [100], suggesting the potential beneficial effects of NMDA receptor antagonists in the treatment of AADC-DY.

Erythropoietin

An additional compound with possible beneficial effects in AADC-DY is the hypoxia-inducible growth factor erythropoietin (EPO). EPO has a role in hematopoiesis and the recombinant human EPO is used for the treatment of anemia. However, EPO and its receptor are also found in the brain, regulating cognition, neurogenesis, and neuroplasticity, while undergoing upregulation upon increase in oxygen demand and brain injury [257]. In vitro studies show that EPO reduced L-dopa-induced oxidative stress and caspase-3, and improved neuronal cell survival [226]. Furthermore, the systemic administration of the recombinant human EPO crosses the BBB, stimulating striatal dopamine release [227]. Similar results were observed in the PD model where EPO prevented neurodegeneration in the nigrostriatal dopaminergic system, improving cognitive function [228]. A clinical study of PD patients treated with intranasally administered EPO reports encouraging results [258]. Whether such an approach combined with other modalities could improve the neuropathology associated with AADC-DY warrants further investigations.

Concluding Remarks

We have extensively discussed oxidative stress in AADC-DY as an ignition for the downstream pathophysiological processes culminating to mitochondrial dysfunction,

neuroinflammation, and neuronal injury and death. We believe that the heterogeneity in the clinical outcomes of established treatments and AAV2-mediated hDDC gene transfer in AADC-DT subjects may be related in part to the progression of the disease where the chronic presence of several upregulated metabolites leads to irreversible damage of neurons, compromising their functional integrity.

The new concept of AADC-DY as a neurodegenerative disease opens a path for additional treatment modalities to reduce the levels of the upregulated metabolites, and or minimize the downstream pathophysiological processes. This in turn would generate a more hospitable environment, where the clinical efficacy of AADC-DY treatments, aimed at restoring neurotransmitters' synthesis and or function, would be enhanced. These additional treatment modalities include the use of COMT inhibitors, antioxidants, and neuroprotective agents such as H2S, ALA, EPO, and vitamins; sulfur-containing compounds such as SAM, methionine, NAC, and 3-MP, able to restore cellular methylation status and enhance cellular antioxidant capacity; antioxidant enzymes such as GSH, catalase, and SOD; and NMDA receptor antagonists with the potential to minimize metabolite-induced excitotoxicity. The use of these treatment modalities as single or in combination, early in the course of the disease, would minimize injury, hence preserving neuronal function. These therapies are not limited to AADC-DY but can be effective in other pathological states, where a mutation has the potential to lead to the accumulation of prooxidants and neurotoxic metabolites. Several disorders of vitamin B6 metabolism fall into this category.

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Declarations

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