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Influence of glutathione on the oxidation chemistry of 5-*S*-cysteinyldopamine: potentially neuroprotective reactions of relevance to Parkinson's disease

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Abstract—In recent reports from this laboratory we have hypothesized that a key step underlying the degeneration of pigmented dopaminergic neurons in the substantia nigra pars compacta (SN_c) in Parkinson's disease is an accelerated rate of oxidation of intraneuronal dopamine in the presence of L-cysteine (CySH) to form initially 5-*S*-cysteinyldopamine (5-*S*-CyS-DA). 5-*S*-CyS-DA, however, is more easily oxidized than dopamine in a reaction which leads to the dihydrobenzothiazine (DHBT) 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1), a putative endogenously-formed metabolite that may be responsible for inhibition of mitochondrial complex I and α-ketoglutarate dehydrogenase, characteristic defects in the parkinsonian SN_c. In this investigation it is demonstrated that glutathione (GSH) dramatically attenuates the oxidative transformation of 5-*S*-CyS-DA into DHBT-1 by two major pathways. In one pathway GSH displaces the cysteinyl residue from the *o*-quinone proximate oxidation product of 5-*S*-CyS-DA forming the corresponding glutathionyl conjugate that is attacked by GSH, to form 2,5-di-*S*-glutathionyldopamine, or by released CySH to give 2-*S*-cysteinyl-5-*S*-glutathionyldopamine. The former is the precursor of 2,5,6-tris-*S*-glutathionyldopamine, a major reaction product. However, intramolecular cyclization of the *o*-quinone proximate product of 2-*S*-cysteinyl-5-*S*-glutathionyldopamine is the first step in a pathway leading to glutathionyl conjugates of 8-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-5). The second pathway involves nucleophilic addition of GSH to the *o*-quinone proximate oxidation product of 5-*S*-CyS-DA forming 2-*S*-glutathionyl-5-*S*-cysteinyldopamine the precursor of a number of glutathionyl conjugates of DHBT-1. These results raise the possibility that strategies which elevate intraneuronal levels of GSH in dopaminergic SN_c cells in Parkinson's disease patients may block formation of the putative mitochondrial toxin DHBT-1 and hence be neuroprotective.

1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative brain disorder after Alzheimer's disease, afflicts more than one million, usually elderly, Americans. The cardinal symptoms of PD are resting tremor, muscular rigidity and therefore difficulties with movement. The degeneration of neuromelanin-pigmented dopaminergic neurons in the substantia nigra pars compacta (SN_c) and consequent massive decrements of dopamine (DA) in the striatum accounts for these motor symptoms of PD.[1] The pathological (neurotoxic) processes that cause the degeneration of dopaminergic SN_c cells in PD include decreased activities of mitochondrial complex I [NADH-coenzyme Q (CoQ) reductase][2] and α-ketoglutarate dehydrogenase (α-KGDH),[3] the latter being an enzyme complex that provides reducing equivalents as succinate to complex II. The complex I defect is not accompanied by altered complex II–IV activities, is specific to the SN_c and exclusive to PD.[2a] Combined impairments of complex I and α-KGDH activities with resultant reduced mitochondrial production of adenosine 5′-triphosphate (ATP), essential for many metabolic processes, therefore appear to be key factors involved in the pathogenesis of PD. However, the fundamental causes of these mitochondrial enzyme defects and SN_c cell death in PD are a mystery.

Oxidative stress, i.e. damage caused by excessive formation of cytotoxic oxygen free radical species, is widely believed to be an important pathogenic factor in PD.[1a] Indeed, at advanced stages of the disorder increased nigral levels of oxidation products of lipids, proteins, DNA and RNA,[4] a massive decrease of the antioxidant glutathione (GSH)[5] and increased levels of iron[6] all appear to provide support for the oxidative stress hypothesis for the pathogenesis of PD. Furthermore, activities of cytoplasmic and mitochondrial superoxide dismutase (SOD) are increased in the parkinsonian SN_c.[7] Since no mutations for SOD genes have been found in PD,[8] the rise of SOD activity suggests an adaptive change in response to elevated superoxide ($O_2^{\cdot-}$) generation. 3-Nitrotyrosine immunoreactivity associated with cytoplasmic proteins in remaining melanized SN_c cells in PD implies intraneuronal generation of peroxynitrite ($ONOO^-$).[9] This, in turn, is indicative of the reaction of elevated intraneuronal

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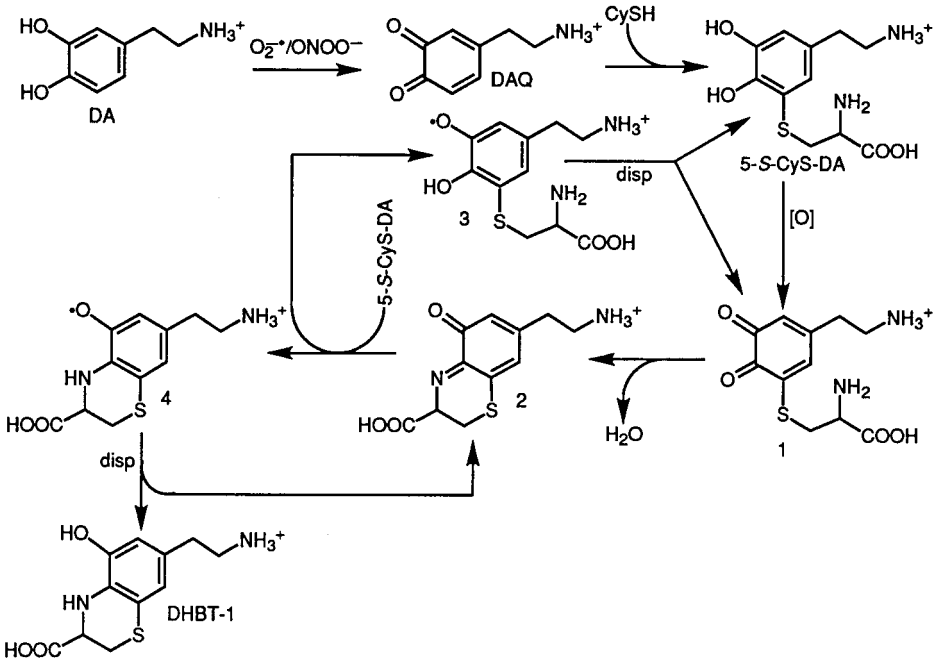
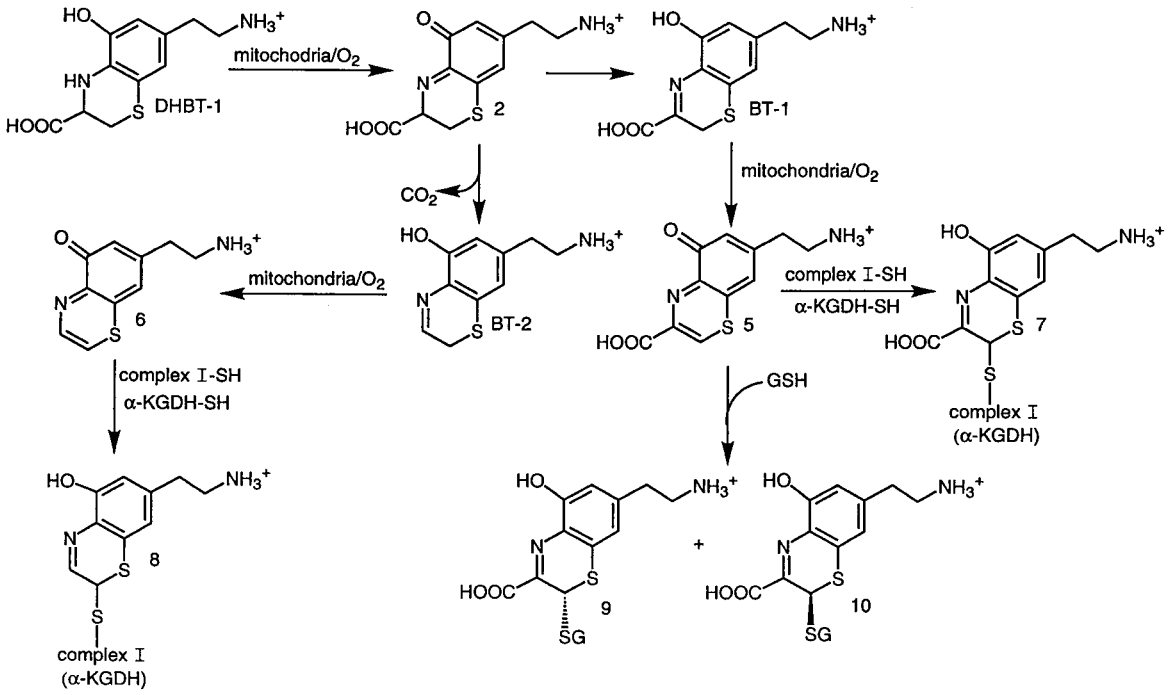


Scheme 1.

$O_2^{\cdot-}$ with nitric oxide ($NO^{\cdot}$) and, hence, activation of neuronal nitric oxide synthase (nNOS).[10] The preceding observations raise the possibility that elevated intraneuronal production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) may be responsible for the mitochondrial complex I and α-KGDH defects and SN_c cell death in PD. However, in vivo, oxidative stress evoked by a number of strategies, including massive depletion of brain GSH, either has no effect on mitochondrial respiratory enzymes[11] or preferentially affects complex IV[12] rather than complex I. Furthermore, $NO^{\cdot}$ selectively inhibits complex IV[13] and $ONOO^-$ irreversibly inhibits many, if not all, mitochondrial respiratory enzymes.[13a,14] Thus, while ROS and RNS appear to play roles in the pathological mechanism underlying the degeneration of dopaminergic SN_c cells in PD they are probably not directly responsible for the complex I or α-KGDH defects.

Another intriguing observation about the parkinsonian SN_c is that the fall of GSH is not accompanied by corresponding increases of glutathione disulfide (GSSG),[5] its expected product of oxidation by ROS and RNS. Furthermore, induction of oxidative stress in cultured DA neurons evokes a compensatory increase of GSH[15] whereas remaining pigmented SN_c cells in the parkinsonian brain contain reduced levels of GSH.[16] Interestingly, patients who may be at early preclinical stages of PD have decreased levels of nigral GSH again without corresponding increases of GSSG. However, the SN_c of these patients exhibit no other evidence for oxidative stress or increased levels of iron and, furthermore, decrements of complex I activity are significantly less than at advanced stages of PD.[4c,17] These observations suggest a connection between the fall of nigral GSH and progressive decrements of complex I activity and SN_c cell death in PD. However, by itself, massive depletion of brain GSH levels leads to neither the degeneration of nigrostriatal DA neurons[18] nor decreased activity of complex I.[11] These observations, together with normal activity of γ-glutamylcysteine synthetase in the parkinsonian SN_c[19] imply that the fall of nigral GSH is not caused by its oxidation to GSSG, impaired mitochondrial production of ATP needed for its biosynthesis (at least at early stages of PD), or a failure of its biosynthetic enzymes.

Based on pathobiochemical changes in the parkinsonian SN_c and those evoked by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a drug that causes the selective degeneration of nigrostriatal DA neurons and, in humans, a syndrome clinically indistinguishable from PD,[20] we recently proposed a new mechanism for the underlying neurotoxic process.[21] The first step in this mechanism was proposed to be a large but transient impairment of DA neuron energy metabolism. In the case of MPTP this would be caused by its active metabolite formed in the brain, 1-methyl-4-phenylpyridinium (MPP^+),[22] a reversible inhibitor of mitochondrial complex I[23] and α-KGDH.[24] The decline of neuron energy metabolism with age,[25] the most robust risk factor for PD, systemic mitochondrial enzyme defects[26] together with periodic exposures to environmental toxicants that interfere with mitochondrial respiratory enzymes,[23b,27] particularly in view of both age- and genetically-based impairments of xenobiotic metabolism,[28] might represent a combination of factors which evoke a profound but transient depletion of neuronal energy in the parkinsonian SN_c. This energy impairment with resultant depolarization of both the neuronal and mitochondrial membranes would mediate a massive release of DA[29] together with both cytoplasmic and mitochondrial GSH.[30] The profound DA neuron energy impairment and relief of the Mg^{2+} block of *N*-methyl-D-aspartate (NMDA) receptors would potentiate activation of these receptors by basal extracellular levels of the excitatory amino acid (EAA) neurotransmitters L-glutamate (Glu), L-aspartate (Asp) and L-cysteine (CySH) with



Scheme 2.

resultant influx of Ca^{2+} and excitotoxicity,[31] a process that mediates neuronal $O_2^{\cdot-}$ generation in excess of the scavenging capacity of SOD[32] and activation of nNOS with resultant $NO^{\cdot}$ and thence $ONOO^-$ production.[33] $O_2^{\cdot-}$, $NO^{\cdot}$ and $ONOO^-$ can all cross the neuronal membrane and release low molecular weight Fe^{2+} from iron-containing proteins.[34] In the presence of extracellular H_2O_2 and ascorbate, such mobilized Fe^{2+} would be expected to catalyze extracellular hydroxyl radical ($HO^{\cdot}$) formation by well-known Fenton/Haber–Weiss chemistry.[35] Furthermore, decomposition of $ONOO^-$ directly forms $HO^{\cdot}$.[36] Indeed, there is compelling evidence that MPTP/MPP^+ and many other energy-depleting insults that lead to the degeneration of nigrostriatal DA neurons mediate generation of extracellular $HO^{\cdot}$.[37] Such extracellular (and intraneuronal) $HO^{\cdot}$ and $ONOO^-$ undoubtedly contribute to damage to lipids, proteins and nucleic acids. However, we have provided evidence that extracellular $HO^{\cdot}$ may be primarily scavenged by GSH initially released from DA neurons.[19] This $HO^{\cdot-}$ mediated oxidation and depletion of extracellular GSH would then be expected to trigger release of the tripeptide from glia as a defensive mechanism to protect neighboring neurons against $HO^{\cdot}$-mediated damage.[19,38]

When the dopaminergic neuron energy impairment begins to subside, increasing ATP production would initiate reinstatement of the Mg^{2+} block of NMDA receptors, decrease Ca^{2+} influx and thus attenuate neuronal $O_2^{\cdot-}$, $NO^{\cdot}$, $ONOO^-$ and hence extracellular $HO^{\cdot}$ generation. More importantly, however, increasing ATP production would initiate reuptake of DA that is readily oxidized by and hence would scavenge both $O_2^{\cdot-}$[39] and $ONOO^-$ (without nitration)[40] and thus completely block extracellular $HO^{\cdot}$ formation. Consequently, the $HO^{\cdot-}$ mediated oxidation of extracellular GSH would cease.

In order to replenish intraneuronal GSH, released and oxidized during the energy impairment, necessitates its continued release from glia. However, neurons are unable to import GSH. Rather, extracellular GSH, released from glia, is first degraded by γ-glutamyl transpeptidase (γ-GT) which transfers the glutamyl residue to acceptor amino acids/peptides or water giving the respective γ-glutamyl derivative or Glu, respectively, and cysteinylglycine (CysGly). CysGly is then hydrolyzed by dipeptidases (DP) to L-cysteine (CySH) and L-glycine (Gly).[41] CySH and CysGly are then translocated into neurons to provide CySH, for GSH biosynthesis.[42] The γ-GT/DP-mediated degradation of GSH is exclusively extracellular.[43] Thus, as the dopaminergic neuron energy impairment begins to subside, the release of glial GSH and its degradation by γ-GT/DP would result in elevation of extracellular CySH (and possibly CysGly), required for intraneuronal GSH biosynthesis, in addition to Gly and may contribute to the delayed elevation of extracellular Glu evoked by MPTP/MPP^+.[44] Furthermore, during this period of recovering but still reduced neuronal ATP production the Mg^{2+} block of NMDA receptors would remain partially relieved and permit continued activation of these receptors by elevated extracellular levels of Glu and CySH with resultant $O_2^{\cdot-}$, $NO^{\cdot}$ and $ONOO^-$ generation and oxidation of DA as it returns to dopaminergic neurons, in the presence of translocated CySH.

The $O_2^{\cdot-}$/$ONOO^-$-mediated oxidation of intraneuronal DA forms dopamine-*o*-quinone (DAQ) which reacts avidly with CySH to give initially 5-*S*-cysteinyldopamine (5-*S*-CyS-DA) (Scheme 1).[39,44] Thus, instead of being utilized for intraneuronal GSH synthesis, translocated CySH would be consumed by DAQ demanding continued release of glial GSH and its degradation by γ-GT/DP to Glu, Gly and

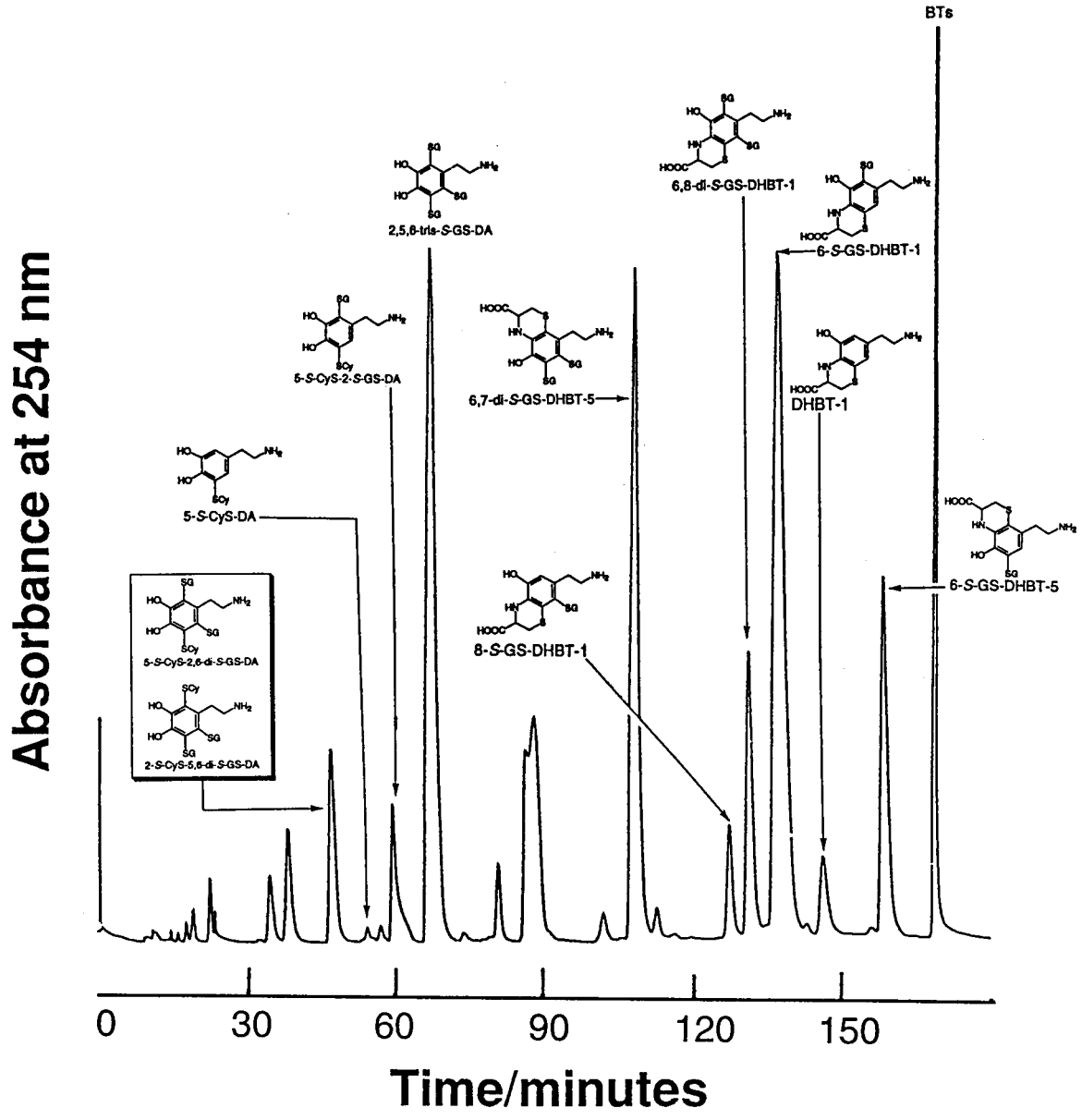


Figure 1. HPLC chromatogram (method I) of the product solution formed following controlled potential electro-oxidation of 1 mM 5-*S*-CyS-DA in the presence of 4 mM GSH in pH 7.4 phosphate buffer (μ=0.2) at 50 mV for 60 min. Injection volume: 10 mL.

CySH with consequent NMDA receptor activation and hence intraneuronal $O_2^{\cdot-}$/$ONOO^-$-mediated oxidation of DA in the presence of CySH and 5-*S*-CyS-DA formation. Indeed, 5-*S*-CyS-DA, normally a very minor metabolite of DA, becomes a major metabolite in the parkinsonian SN_c as evidenced by a dramatic increase of the 5-*S*-CyS-DA/DA concentration ratio.[39,46] However, 5-*S*-CyS-DA is appreciably more easily oxidized than DA, from which it derives.[45] The first step in this reaction involves oxidation of 5-*S*-CyS-DA to *o*-quinone **1** that undergoes a very rapid intramolecular cyclization to *o*-quinone imine **2** (Scheme 1). Intermediate **2** then chemically oxidizes 5-*S*-CyS-DA forming radicals **3** and **4**, the former disproportionating to 5-*S*-CyS-DA and **1** and the latter to **2** and the dihydrobenzothiazine (DHBT) 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1).

In vitro, DHBT-1 can be accumulated by intact rat brain mitochondria and evoke irreversible inhibition of complex I, but not complexes II–IV, and α-KGDH.[47] The selectivity of DHBT-1 for these mitochondrial enzyme complexes derives from its oxidation, catalyzed by a presently unknown constituent of the inner mitochondrial membrane, initially to *o*-quinone imine **2** that rapidly rearranges to the benzothiazine (BT) metabolites BT-1 and BT-2 (Scheme 2).[47] BT-1 and BT-2 are further oxidized, again in a reaction catalyzed by the mitochondrial membrane, to highly electrophilic quinone imines **5** and **6** that have been proposed to covalently bind to active site cysteinyl -SH residues of NADH-CoQ reductase and α-KGDH as conceptualized in structures **7** and **8** (Scheme 2), respectively, thus evoking irreversible inhibition of these enzyme complexes. These results have led us to suggest that DHBT-1 and its oxidative metabolites BT-1 and BT-2 may be endotoxicants responsible for the complex I and α-KGDH defects in the parkinsonian SN_c. Interestingly, greater than equimolar excesses

of GSH completely block the irreversible inhibition of NADH-CoQ reductase and α-KGDH by DHBT-1, BT-1 and BT-2, without affecting the rate of their mitochondrial membrane-catalyzed oxidation, with resultant formation of new metabolites that include the epimeric glutathionyl conjugates **9** and **10** (Scheme 2). These observations provide support for the notion that electrophilic intermediates **5** and **6**, formed by the mitochondrial membrane-catalyzed oxidation of BT-1 and BT-2, respectively, are responsible for covalent modification of active site cysteinyl residues of NADH-CoQ reductase and α-KGDH. However, they also imply that excess intramitochondrial GSH should block the irreversible inhibition of complex I and α-KGDH by DHBT-1, BT-1 and BT-2. This points to the importance of the release of intraneuronal GSH during the DA neuron energy impairment. Mitochondrial membranes also catalyze the oxidation of 5-*S*-CyS-DA forming DHBT-1 that subsequently irreversibly inhibits NADH-CoQ reductase and α-KGDH by the pathway conceptualized in Scheme 2. However, unlike DHBT-1, 5-*S*-CyS-DA does not inhibit complex I respiration when incubated with intact rat brain mitochondria suggesting it is unable to easily cross the mitochondrial membrane.[47a,b] Thus, in order to form DHBT-1, which can be transported into mitochondria, it would be necessary for 5-*S*-CyS-DA to be oxidized by $O_2^{\cdot-}$/$ONOO^-$ in the cytoplasm of dopaminergic neurons. Reports that elevation of brain GSH or other thiol levels attenuates the dopaminergic neurotoxicity of MPTP[48] raises the possibility that GSH might interfere with the oxidation of cytoplasmic 5-*S*-CyS-DA to the mitochondrial toxin DHBT-1. This communication represents the first step aimed at exploring this possibility by investigating, in vitro, the influence of excess GSH on the oxidation chemistry of 5-*S*-CyS-DA. Controlled potential electro-oxidation of 5-*S*-CyS-DA was employed as a convenient method to generate *o*-quinone **1** in the presence of free GSH.

2. Results

2.1. Influence of GSH on the oxidation chemistry of 5-*S*-CyS-DA

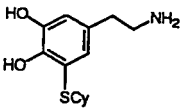
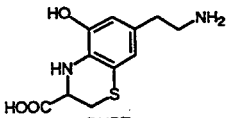
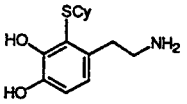
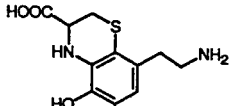
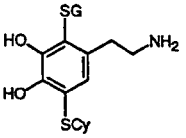
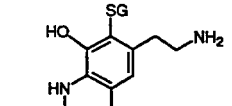
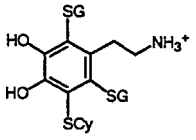
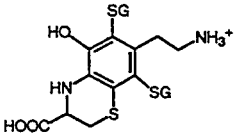
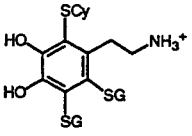
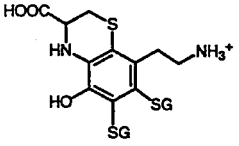
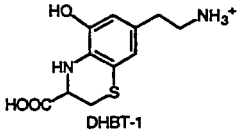
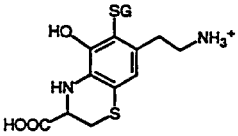
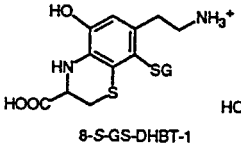
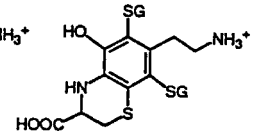
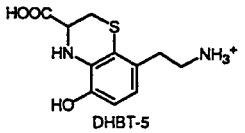
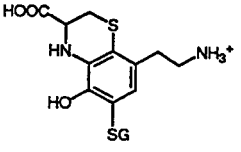
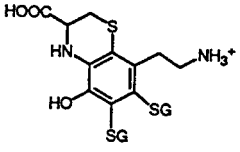
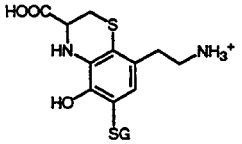
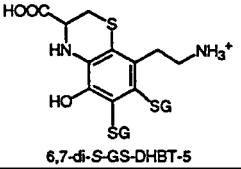
Previous studies have demonstrated that during the early stages of controlled potential electro-oxidation of 5-*S*-CyS-DA (1 mM) at 50 mV in pH 7.4 phosphate buffer DHBT-1 is formed in almost quantitative yield[45b] by the reaction pathway shown in Scheme 1. At longer reaction times DHBT-1 is further oxidized to benzothiazines BT-1 and BT-2 (Scheme 2)[47b,49] which give a characteristic yellow color to the reaction solution. HPLC analysis of the product solution formed throughout a controlled potential electro-oxidation (50 mV; pH 7.4) of 5-*S*-CyS-DA (1 mM) in the presence of GSH (4 mM) showed that DHBT-1 was always a very minor product that at no time accounted for more than 5% of the 5-*S*-CyS-DA oxidized. An HPLC chromatogram of the product solution formed after oxidation of 5-*S*-CyS-DA in the presence of GSH for 60 min, a point when virtually all 5-*S*-CyS-DA had been oxidized, is presented in Fig. 1 and demonstrates that DHBT-1 is indeed a very minor reaction product. The major products of the reaction were 2,5,6-tris-*S*-GS-DA, 6,7-di-*S*-GS-DHBT-5 and 6-*S*-GS-DHBT-1. More minor products were 6-*S*-GS-DHBT-5, 8-*S*-GS-DHBT-1, 6,8-di-*S*-GS-DHBT-1, 5-*S*-CyS-2-*S*-GS-DA, 2-*S*-CyS-5,6-di-*S*-GS-DA and 5-*S*-CyS-2,6-di-*S*-GS-DA. Attempts to isolate products responsible for other small chromatographic peaks present in Fig. 1 were unsuccessful either because several very minor compounds eluted under one peak or the compounds were formed in such low yield that insufficient material could be isolated for subsequent spectroscopic structure elucidation. The solution that eluted under the large peak (labeled BTs) at the end of the chromatogram shown in Fig. 1 was deep yellow. Subsequent studies revealed that this peak was due to a rather large number of secondary BT products formed by oxidations of various DHBTs. However, attempts to isolate and identify these compounds have not yet been successful.

Spectroscopic evidence in support of the structures of previously unreported compounds resulting from the oxidation of 5-*S*-CyS-DA in the presence of GSH is provided in the Experimental. However, additional evidence for several key structures was provided by chemical experiments summarized in Table 1. For example, the structure of 5-*S*-CyS-2-*S*-GS-DA was confirmed on the basis that the major initial product of its controlled potential electro-oxidation (50 mV; pH 7.4) was 6-*S*-GS-DHBT-1, a conjugate that is also the major initial product of oxidation of DHBT-1 in the presence of GSH. Similarly, controlled potential electro-oxidation of 5-*S*-CyS-2,6-di-GS-DA forms 6,8-di-*S*-GS-DHBT-1, also formed by oxidation of DHBT-1 and 6-*S*-GS-DHBT-1 in the presence of free GSH. The initial major product of controlled potential electro-oxidation 2-*S*-CyS-5,6-di-*S*-GS-DA is 6,7-di-*S*-GS-DHBT-5, also formed by oxidation of DHBT-5 in the presence of GSH. These observations unequivocally confirm that the cysteinyl residue in 2-*S*-CyS-5,6-di-*S*-GS-DA is indeed located at C(2).

2.2. Reaction pathways

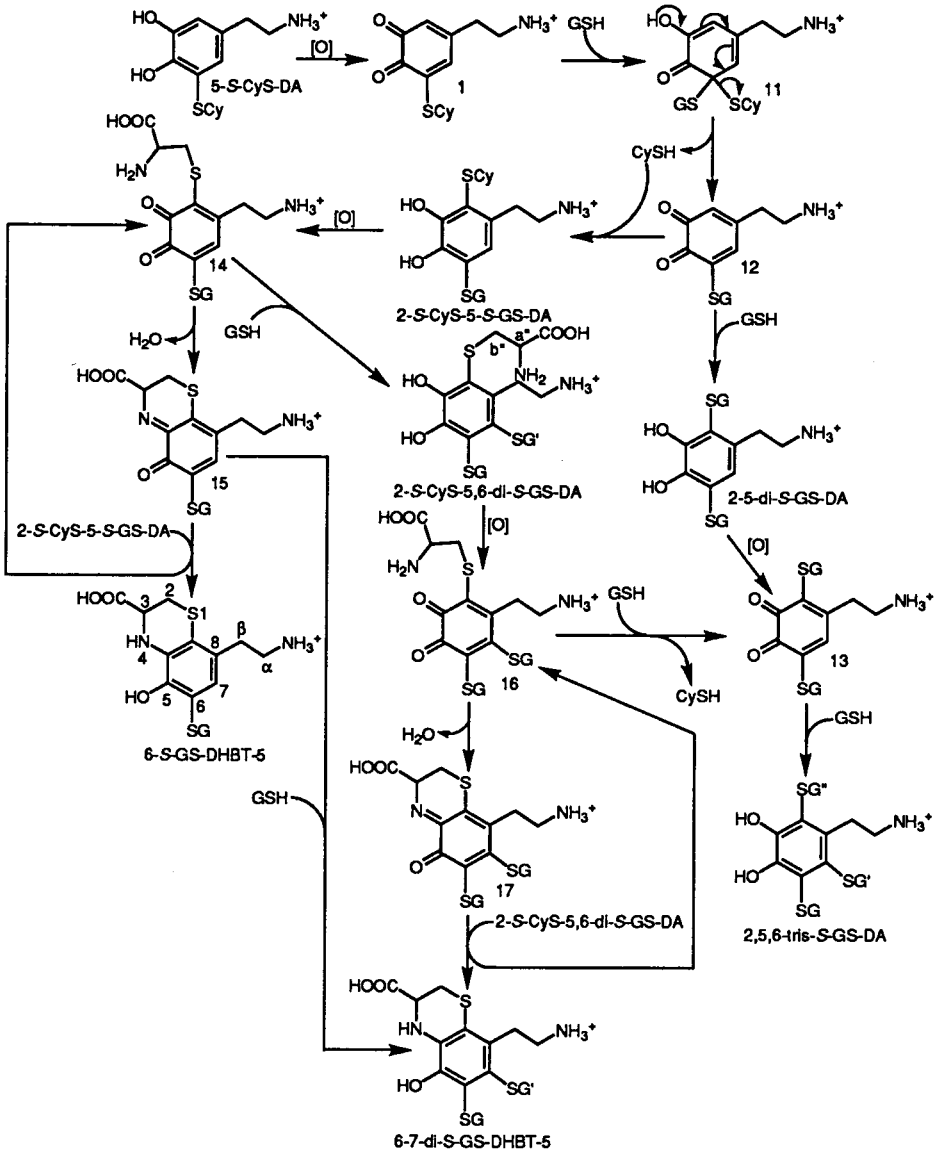
Formation of 2-*S*-CyS-5,6-di-*S*-GS-DA, 2,5,6-tris-*S*-GS-DA, 6-*S*-GS-DHBT-5 and 6,8-di-*S*-GS-DHBT-5 clearly indicate that GSH can displace the cysteinyl residue of 5-*S*-CyS-DA under oxidative conditions and that the released CySH is an active participant in subsequent reactions. Thus, it appears that nucleophilic addition of GSH to *o*-quinone **1** transiently forms intermediate **11** followed by expulsion of free CySH to give the 5-*S*-glutathionyl-substituted *o*-quinone **12** (Scheme 3). Overall, this represents a nucleophilic displacement reaction. Subsequent nucleophilic addition of GSH to **12** would then generate, predominantly, 2,5-di-*S*-GS-DA.[50] That 2,5-di-*S*-GS-DA is not detected as a significant product of oxidation of 5-*S*-CyS-DA in the presence of GSH probably reflects the fact that this is, by far, the most easily oxidized glutathionyl conjugate of DA.[50] This reaction oxidizes 2,5-di-*S*-GS-DA to *o*-quinone **13** which is further substituted by GSH to give 2,5,6-tris-*S*-GS-DA, a major reaction product (Fig. 1). Nucleophilic addition of CySH, released from putative intermediate **11**, to *o*-quinone **12** then generates 2-*S*-CyS-5-*S*-GS-DA (Scheme 3). The latter mixed conjugate was not observed as a significant reaction product presumably owing to its facile oxidation to *o*-quinone **14**. Nucleophilic addition of GSH to **14** then accounts for formation of 2-*S*-CyS-5,6-

Table 1. Products of controlled potential electro-oxidation of cysteinyl/glutathionyl conjugates of dopamine and DHBTs at pH 7.4 (electrolyzed at 50 mV in pH 7.4 phosphate buffer (μ=0.2) in the presence of the indicated concentration of GSH)

Compound[a]	free GSH concn./mM	Initial products[b] major	Initial products[b] minor
 5-*S*-CyS-DA	0	 DHBT-1	
 2-*S*-CyS-DA	0	 DHBT-5	
 5-*S*-CyS-2-*S*-GS-DA	0	 6-*S*-GS-DHBT-1	
 5-*S*-CyS-2,6-di-*S*-GS-DA	0	 6,8-di-*S*-GS-DHBT-1	
 2-*S*-CyS-5,6-di-*S*-GS-DA	0	 6,7-di-*S*-GS-DHBT-5	
 DHBT-1	4	 6-*S*-GS-DHBT-1	 8-*S*-GS-DHBT-1  6,8-di-*S*-GS-DHBT-1
 DHBT-5	4	 6-*S*-GS-DHBT-5	 6,7-di-*S*-GS-DHBT-5
 6-*S*-GS-DHBT-5	4	 6,7-di-*S*-GS-DHBT-5	

[a] Each compound had an initial concentration of 1 mM.

[b] Oxidation reactions were terminated after ≤20 min, products separated by preparative HPLC method I and after isolation spectroscopically characterized (UV, ^{1}H NMR, FAB-MS).



Scheme 3.

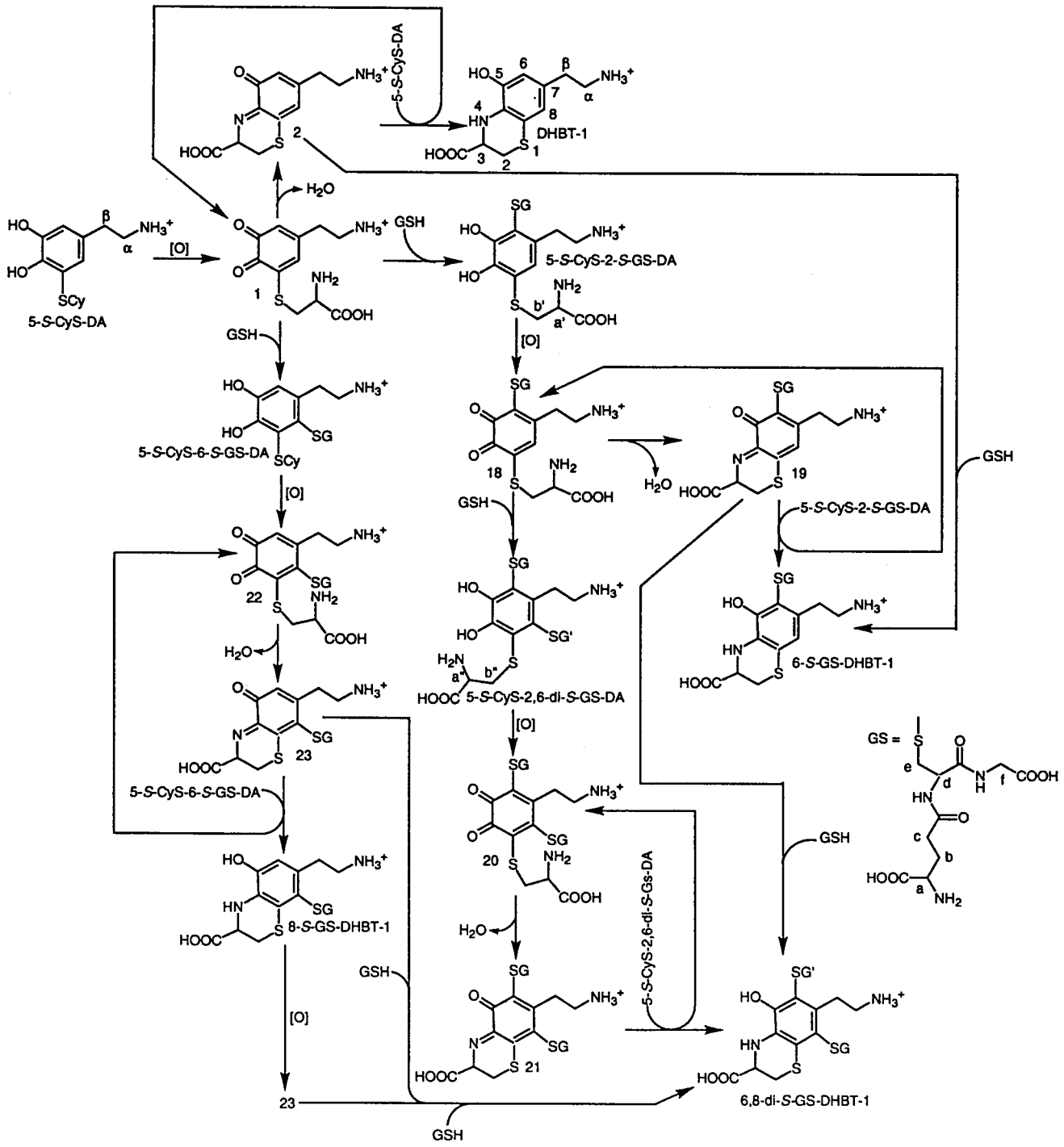
di-*S*-GS-DA. However, intramolecular cyclization of **14** with resultant formation of **15** and reaction of this *o*-quinone imine with 2-*S*-CyS-5-*S*-GS-DA (and probably other cysteinyl conjugates of DA) then accounts for formation of 6-*S*-GS-DHBT-5[†] by a mechanism similar to that discussed in more detail in connection with DHBT-1 formation from 5-*S*-CyS-DA in Scheme 1. Nucleophilic addition of GSH to **15** provides one route to 6,7-di-*S*-GS-DHBT-5. Additionally, intramolecular cyclization of *o*-quinone **16**, formed by oxidation of 2-*S*-CyS-5,6-di-*S*-GS-DA, to **17** and its subsequent reaction with 2-*S*-CyS-5,6-di-*S*-GS-DA provides a second route to 6,7-di-*S*-GS-DHBT-5.

A second major route by which *o*-quinone **1**, the proximate oxidation product of 5-*S*-CyS-DA, is consumed is initiated by nucleophilic addition of GSH to form 5-*S*-CyS-2-*S*-GS-DA (Scheme 4). This relatively minor product is further oxidized to *o*-quinone **18**.

Intramolecular cyclization of **18** with resultant formation of *o*-quinone imine **19** that is reduced by 5-*S*-CyS-2-*S*-GS-DA (and probably other conjugates of DA) then accounts for formation of 6-*S*-GS-DHBT-1, a major reaction product. Nucleophilic addition of GSH to **18** accounts for formation of 5-*S*-CyS-2,6-di-*S*-GS-DA. Further oxidation of the latter mixed conjugate to **20** followed by intramolecular cyclization to **21** and its chemical reduction then provides one pathway to 6,8-di-*S*-GS-DHBT-1.

Nucleophilic addition of GSH to *o*-quinone imine **19** provides a second pathway to 6,8-di-*S*-GS-DHBT-1. The initial step leading to formation of 8-*S*-GS-DHBT-1 must involve nucleophilic addition of GSH to *o*-quinone **1**. Subsequent oxidation of the resultant 5-*S*-CyS-2-*S*-GS-DA

[†] DHBT-5,8-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid, is formed by oxidation of 2-*S*-cysteinyldopamine in a reaction analogous to that by which 5-*S*-CyS-DA is transformed into DHBT-1 (Scheme 1). The abbreviation DHBT-5 is for consistency with earlier reports from this laboratory.[45a,b]



Scheme 4.

leads to *o*-quinone **22** and then *o*-quinone imine **23** that is chemically reduced by 5-*S*-CyS-6-*S*-GS-DA to 8-*S*-GS-DHBT-1, a relatively minor reaction product. Nucleophilic addition of GSH to *o*-quinone imine **23** provides a third pathway to 6,8-di-*S*-GS-DHBT-1 (Scheme 4).

Although DHBT-1 is formed as only a minor product during the oxidation of 5-*S*-CyS-DA in the presence of GSH, nucleophilic addition of GSH to its precursor (Scheme 1) and proximate oxidation product (Scheme 2), *o*-quinone imine **2**, probably contributes to formation of 6-*S*-GS-DHBT-1 and thence 6,8-di-*S*-GS-DHBT-1 (Scheme 4).

3. Discussion

The hypothesis we have proposed for a mechanism that might contribute to the degeneration of dopaminergic neurons in PD and evoked by neurotoxins such as MPTP is based on two key steps. The first is a large but transient

impairment of dopaminergic neuron energy metabolism. The resultant profound depolarization of the mitochondrial and neuronal membranes then mediates a massive release of DA together with both cytoplasmic and mitochondrial GSH. The second key step occurs as the neuron energy impairment begins to subside and involves the $O_2^{\cdot-}$/$ONOO^-$-mediated oxidation of DA, as it returns via its transporter into the cytoplasm of its parent neurons, to DAQ which reacts with translocated CySH to give first 5-*S*-CyS-DA that is then oxidized to DHBT-1 (Scheme 1). Based on the results of in vitro studies, DHBT-1 can be accumulated by mitochondria and catalytically oxidized at the inner membrane forming electrophilic intermediates such as **5** and **6** which have been proposed to covalently bind to and modify active site cysteinyl residues of NADH-CoQ reductase and α-KGDH causing their irreversible inhibition (Scheme 2). Thus, DHBT-1 may be an endogenously formed oxidative metabolite of 5-*S*-CyS-DA that contributes to the mitochondrial complex I[2] and α-KGDH[3] defects in the parkinsonian SN_c and the delayed inhibition of complex I evoked by MPTP[51] leading to the death of dopaminergic neurons. However, it must be emphasized that although the 5-*S*-CyS-DA/DA concentration ratio rises dramatically in the parkinsonian SN_c,[46] suggestive of an increased rate of DA oxidation and increased availability of CySH, there is presently no direct evidence that DHBT-1 or its oxidative metabolites are responsible for the complex I and α-KGDH defects. Indeed, the fact that DHBT-1, BT-1 and BT-2 are catalytically oxidized to electrophilic intermediates that covalently bind to nucleophilic sites on mitochondrial proteins (Scheme 2) makes it somewhat unlikely that these putative metabolites could be detected as free bases.

It has recently been demonstrated that greater than equimolar concentrations of GSH blocks the DHBT-1-mediated irreversible inhibition of NADH-CoQ reductase and α-KGDH by scavenging electrophilic intermediates such as **5** and **6** (Scheme 2).[47] It is for this reason that release of mitochondrial GSH during the dopaminergic energy impairment represents an important step in the hypothesized neurotoxic mechanism. The results of the present investigation suggest that significant concentrations of GSH in the cytoplasm of dopaminergic neurons as the energy impairment begins to subside would attenuate or block formation of DHBT-1 and hence be neuroprotective. In principle, such neuroprotection could result from several reaction pathways. Thus, cytoplasmic GSH would be expected to compete with translocated CySH for DAQ forming 5-*S*-glutathionyldopamine (5-*S*-GS-DA).[50]

Intraneuronal 5-*S*-GS-DA cannot be converted into 5-*S*-CyS-DA because γ-GT, the only mammalian enzyme that can initiate this process, is active only toward extracellular substrates.[43] Nucleophilic displacement of the cysteinyl residue of *o*-quinone **1** by GSH also blocks the pathway leading to DHBT-1 (Scheme 3). Nucleophilic addition of GSH to **1** also blocks DHBT-1 formation (Scheme 4). Similarly, nucleophilic addition of GSH to *o*-quinone imine **2** would also attenuate formation of DHBT-1 (Scheme 4). Thus, based on the present results, significant cytoplasmic concentrations of GSH would be expected to block formation of DHBT-1. Furthermore, even if DHBT-1 is formed in the cytoplasm of dopaminergic neurons, mitochondrial GSH could provide a second line of defense by blocking its inhibition of NADH-CoQ reductase and α-KGDH as conceptualized by the reactions in Scheme 2.

The oxidation of 5-*S*-CyS-DA in the presence of GSH clearly results in formation of a large number of glutathionyl and mixed conjugates of DA and glutathionyl conjugates of DHBT-1 and DHBT-5. At the present time, it is not known whether these compounds include inhibitors of mitochondrial enzyme complexes. Nevertheless, reports that elevation of brain GSH or, indeed, other thiols attenuates the dopaminergic neurotoxicity of MPTP[48] or other energy-depleting insults that normally lead to the degeneration of DA neurons[52] provide some support for the suggestion that neuroprotection may be the consequence of reaction pathways of the type summarized in Schemes 2–4. Indeed, it is of interest that one study with early PD patients has demonstrated that GSH may not only be of therapeutic use but might also retard the progression of the neurodegenerative processes.[53]

The concentration of 5-*S*-CyS-DA employed in this investigation may be physiologically relevant. Thus, DA concentrations within striatal axon varicosities and SN_c cell bodies are approximately 50 mM and >1 mM, respectively[54] and vesicular concentrations of this neurotransmitter have been estimated to be 0.8 M or higher.[55] Intraneuronal and intraglial concentrations of GSH have been estimated to be 2.5 and 3.8 mM, respectively.[56] Exocytotic release of DA has been estimated to lead to an instantaneous extracellular concentration of 70 μM[55b] and very much larger concentrations of the neurotransmitter[29] and GSH[21] are released in response to a large impairment of dopaminergic neuron energy metabolism. Thus, as the energy impairment begins to subside, sufficient DA and CySH, formed by the γ-GT/DP-mediated degradation of GSH that continues to be released from glia,[21] should be available to form millimolar instantaneous concentrations of 5-*S*-CyS-DA. The fact that measured concentrations of 5-*S*-CyS-DA in the parkinsonian SN_c are very low[39,46] may be the result of its further oxidation to DHBT-1 and thence to electrophilic metabolites responsible for irreversible inhibition of complex I and α-KGDH.

4. Experimental

4.1. Chemicals

Glutathione (GSH), L-cysteine (CySH), and DA (hydrochloride salt) were obtained from Sigma (St. Louis, MO). 5-*S*-CyS-DA, 2-*S*-CyS-DA, 5-*S*-glutathionyldopamine (5-*S*-GS-DA), 2,5,6-tris-*S*-glutathionyldopamine (2,5,6-tris-*S*-GS-DA), DHBT-1 and DHBT-5 were synthesized and purified as described previously.[45]

4.2. Electrochemistry

Controlled potential electrolyses employed a Princeton Applied Research Corp. (Princeton, NJ) model 173 potentiostat. A three-compartment cell was used in which the

working, counter, and reference electrode compartments were separated with a Nafion membrane (type 117, DuPont Co., Wilmington, DE). The working electrode consisted of several plates of pyrolytic graphite having a total surface area of approximately 180 cm^2. The counter electrode was platinum gauze, and the reference electrode was a saturated calomel electrode (SCE). The solution in the working electrode compartment was vigorously bubbled with nitrogen and stirred with a Teflon-coated magnetic stirring bar. All potentials are referred to the SCE at ambient temperature (22±2°C).

4.3. Spectroscopy

NMR spectra were recorded on a Varian (Palo Alto, CA) Inova 400 spectrometer. Low- and high-resolution fast atom bombardment mass spectrometry (FAB-MS) employed a VG Instruments (Manchester, UK) ZAB-E spectrometer. UV-visible spectra were recorded on a Hewlett-Packard (Palo Alto, CA) model 8452A diode array spectrophotometer.

4.4. High performance liquid chromatography (HPLC)

HPLC employed a Gilson (Middleton, WI) gradient system equipped with dual model 302 pumps (10 mL pump heads), a Rheodyne (Cotati, CA) model 7125 loop injector, and a Waters (Milford, MA) model 440 UV detector set at 254 nm. Three mobile phase solvents were used. Solvent A was prepared by adding concentrated trifluoroacetic acid (TFA) to deionized water until the pH was 2.15. Solvent B was prepared by adding TFA to a 1:1 (v/v) solution of HPLC grade acetonitrile (MeCN) and deionized water until the measured pH was 2.15. Solvent C was prepared by adding TFA to deionized water until the pH was 1.50. A preparative scale reversed phase column (Bakerbond C_{18}, 10 μm, 250×21.2 mm, P.J. Cobert Associates, St. Louis, MO) was used. HPLC method I employed the following mobile phase gradient: 0–20 min, 100% solvent A; 20–160 min, linear gradient to 18% solvent B, 160–164 min, linear gradient to 100% solvent B; 164–176 min, 100% solvent B. HPLC method II employed the following gradient: 0–20 min, 100% solvent C; 20–100 min, linear gradient to 9% solvent B; 100–104 min, linear gradient to 100% solvent B; 104–116 min, 100% solvent B. The flow rate for HPLC methods I and II was 7 mL min^{-1}.

4.5. Oxidation reaction procedure

5-*S*-CyS-DA (1 mM) and GSH (4 mM) were dissolved in 30 mL of phosphate buffer (pH 7.4, μ=0.2) and the initially colorless solution oxidized by controlled potential electrolysis at 50 mV for up to 60 min. The entire resultant pale yellow solution was normally pumped directly onto the preparative reversed phase column and products separated using HPLC method I. The solutions eluted under each chromatographic peak were collected, shell-frozen at −80°C and then freeze-dried. Each product was then dissolved in the minimum volume of deionized water adjusted to pH 2.15 with TFA and purified at least one additional time using HPLC method I. Using HPLC method I, 5-*S*-cysteinyl-2,6-di-*S*-glutathionyldopamine (5-*S*-CyS-2,6-di-*S*-GS-DA) and 2-*S*-cysteinyl-5,6-di-*S*-glutathionyldopamine (2-*S*-CyS-5,6-di-*S*-GS-DA) coeluted (Fig. 1). Thus, using HPLC method I, the eluent containing a mixture of these two compounds was first collected. The solution was subsequently injected onto the preparative reversed phase column and 5-*S*-CyS-2,6-di-*S*-GS-DA and 2-*S*-CyS-5,6-di-*S*-GS-DA separated and purified using HPLC method II.

Spectroscopic and other evidence bearing on the structures of new isolated products are presented below. All ^{1}H NMR assignments were confirmed by two-dimensional correlated spectroscopy experiments.

4.5.1. 2-*S*-Glutathionyl-5-*S*-cysteinyldopamine (2-*S*-GS-5-*S*-CyS-DA). This compound was isolated as a white solid. In the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=304 (sh) and 274 nm. FAB-MS (thioglycerol matrix) gave *m*/*z*=578.1594 (MH^+, 6%, $C_{21}H_{32}N_5O_{10}S_2$; calcd *m*/*z*=578.1591). ^{1}H NMR (400 MHz, D_2O) gave δ: 6.89 (s, 1H, C(6)-H), 4.20(dd, *J*=8.4, 4.4 Hz, 1H, C(d)-H), 3.79(dd, *J*=6.4, 4.8 Hz, 1H, C(a′)-H), 3.69(s, 2H, C(f)-H_2), 3.68 (dd, 13.2, 8.4 Hz, C(a)-H), 3.29(*J*=15.2, 4.8 Hz, 1H, C(b′)-H), 3.24(dd, *J*=15.2, 6.4 Hz, 1H, C(b′)-H), 3.14 (dd, *J*=14.0, 4.4 Hz, 1H, C(e)-H), 3.08–2.88 (m, 5H, C(e)-H, C(β)-H_2, C(α)-H_2), 2.31–2.26 (m, 2H, C(c)-H_2), 2.00–1.88 (m, 2H, C(b)-H_2).

4.5.2. 5-*S*-Cysteinyl-2,6-di-*S*-glutathionyldopamine (5-*S*-CyS-2,6-di-*S*-GS-DA). This compound was isolated as a white solid. In the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=316, 290 (sh), 242 (sh) and 222 nm. FAB-MS (thioglycerol matrix) gave *m*/*z*=883.2289 (MH^+, 7%, $C_{31}H_{47}N_8O_{16}S_3$; calcd *m*/*z*=883.2272). ^{1}H NMR (400 MHz, D_2O) gave δ: 4.23 (dd, *J*=8.4, 4.8 Hz, 2H, C(d)-H, C(d′)-H), 3.86 (t, *J*=5.2 Hz, 1H, C(a″)-H), 3.78 (t, *J*=6.4 Hz, 1H, C(a)-H), 3.77 (t, *J*=6.4 Hz, 1H, C(a′)-H), 3.76 (d, *J*= 18.0 Hz, 1H, C(f)-H), 3.73 (d, *J*=18.0 Hz, 1H, C(f′)-H), 3.70 (d, *J*=18.0 Hz, 1H, C(f)-H), 3.68 (d, *J*=18.0 Hz, 1H, C(f′)-H), 3.48 (dd, *J*=15.2, 5.2 Hz, 1H, C(b″)-H), 3.43–3.33 (m, 2H, C(α)-H_2), 3.14 (dd, *J*=15.2, 5.2 Hz, 1H, C(b″)-H), 3.12 (dd, *J*=14.0, 4.8 Hz, 2H, C(e)-H, C(e′)-H), 3.02 (dd, *J*=14.0, 8.4 Hz, 1H, C(e)-H), 3.01 (dd, *J*=14.0, 8.4 Hz, 1H, C(e′)-H), 2.95–2.91 (m, 2H, C(β)-H_2), 2.35 (t, *J*=7.6 Hz, 4H, C(c)-H2, C(c′)-H_2), 2.01–1.95 (m, 4H, C(b)-H_2, C(b′)-H_2).

4.5.3. 2-*S*-Cysteinyl-5,6-di-*S*-glutathionyldopamine (2-*S*-CyS-5,6-di-*S*-GS-DA). This compound was isolated as a white solid. Dissolved in the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=316, 290 (sh), 242 (sh) and 222 nm. FAB-MS (thioglycerol matrix) gave *m*/*z*=883.2257 (MH^+, 10%,$C_{31}H_{47}N_8O_{16}S_3$; calcd *m*/*z*=883.2272). ^{1}H NMR (400 MHz, D_2O) gave δ: 4.18 (dd, *J*=8.8, 4.8 Hz, 1H, C(d)-H), 4.04 (dd, *J*=8.8, 4.8 Hz, 1H, C(d′)-H), 3.90 (dd, *J*=6.4, 4.8 Hz, 1H, C(a″)-H), 3.75 (t, *J*=6.4 Hz, 1H, C(a)-H), 3.74 (t, *J*=6.4 Hz, 1H, C(a′)-H), 3.74 (d, *J*= 18.0 Hz, 1H, C(f)-H), 3.69 (d, *J*=18.0 Hz, 1H, C(f)-H), 3.65 (s, 2H, C(f′)-H_2), 3.48–3.38 (m, 2H, C(α)-H_2), 3.34 (dd, *J*=14.4, 4.8 Hz, 1H, C(e′)-H), 3.24 (dd, *J*=14.8, 6.4 Hz, 1H, C(b″)-H), 3.20 (dd, *J*=14.4, 4.8 Hz, 1H, C(e)-H), 3.15 (dd, *J*=14.8, 4.8 Hz, 1H, C(b″)-H), 3.06 (dd, *J*=14.4, 8.8 Hz, 1H, C(e′)-H), 3.00 (dd, *J*=14.4,

8.8 Hz, 1H, C(e)-H), 2.93 (t, J=8.0 Hz, 2H, C(β)-H_2), 2.38–2.30 (m, 4H, C(c)-H_2, C(c′)-H_2), 2.01–1.93 (m, 4H, C(b)-H_2, C(b′)-H_2).

4.5.4. 2,5,6-Tris-*S*-glutathionyldopamine (2,5,6-tris-*S*-GS-DA). This compound was isolated as a white solid. Dissolved in the HPLC mobile phase (pH 2.15) it showed UV bands at λ_{max}=312, 290 (sh), 244 (sh) and 222 nm. FAB-MS (thioglycerol matrix) gave m/z=1069.2926 (MH^+, 3%, $C_{38}H_{57}N_{10}O_{20}S_3$; calcd m/z=1069.2913). ^{1}H NMR (400 MHz, D_2O) gave δ: 4.24 (dd, J=8.4, 4.8 Hz, 2H, C(d)-H), C(d′)-H), 4.06 (dd, J=8.4, 4.8 Hz, 1H, C(d″)-H), 3.80 (t, J=6.4 Hz, 1H, C(a)-H), 3.79 (t, J=6.4 Hz, 2H, C(a′)-H), C(a″)-H), 3.76 (d, J=18.0 Hz, 1H, C(f)-H), 3.75 (d, J=18.0 Hz, 1H, C(f′)-H), 3.71 (d, J=18.0 Hz, 1H, C(f)-H), 3.70 (d, J=18.0 Hz, 1H, C(f′)-H), 3.65 (s, 2H, C(f″)-H_2), 3.47–3.35 (m, 2H, C(α)-H_2), 3.32 (dd, J=14.4, 4.8 Hz, 1H, C(e″)-H), 3.18–2.98 (m, 5H, C(e)-H_2), C(e′)-H_2), C(e″)-H), 2.94–2.88 (m, 2H, C(β)-H_2), 2.38–2.34 (m, 6H, C(c)-H_2, C(c′)-H_2, C(c″)-H), 2.03–1.96 (m, 6H, C(b)-H_2, C(b′)-H_2, C(b″)-H_2).

4.5.5. 6-*S*-Glutathionyl-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (6-*S*-GS-DHBT-1). This compound was isolated as a very pale yellow solid. Dissolved in the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=318, 280 (sh) and 244 nm. In pH 7.4 phosphate buffer the UV spectrum showed λ_{max}, nm (log ϵ_{max}, $M^{-1}\,cm^{-1}$) at 320 (3.48), 280 (sh, 3.88) and 250 (4.33), calculated as 6-*S*-GS-DHBT-1.2 TFA. FAB-MS (thioglycerol matrix) gave m/z=560.1467 (MH^+, 34%, $C_{21}H_{30}N_5O_9S_2$; calcd m/z=560.1485). ^{1}H NMR (400 MHz, D_2O) gave δ: 6.51 (s, 1H, C(8)-H), 4.46 (dd, J=4.4, 3.2 Hz, 1H, C(3)-H), 4.24 (dd, J=8.4, 4.4 Hz, 1H, C(d)-H), 3.79 (d, J=18.0 Hz, 1H, C(f)-H), 3.72 (t, J=6.4 Hz, 1H, C(a)-H), 3.71 (J=18.0 Hz, 1H, C(f)-H), 3.19 (dd, J=13.2, 4.4 Hz, 1H, C(2)-H), 3.11 (dd, J=13.2, 3.2 Hz, 1H, C(2)-H), 3.05–2.98 (m, 3H, C(e)-H, C(α)-H_2), 2.93–2.87 (m, 3H, C(e)-H, C(β)-H_2), 2.34–2.20 (m, 2H, C(c)-H_2), 1.98–1.92 (m, 2H, C(b)-H_2).

4.5.6. 8-*S*-Glutathionyl-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (8-*S*-GS-DHBT-1). This compound was a very pale yellow solid. Dissolved in the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=318 and 244 nm. FAB-MS (thioglycerol matrix) gave m/z=560.1475 (MH^+, 17%, $C_{21}H_{30}N_5O_9S_2$; calcd m/z= 560.1485). ^{1}H NMR (400 MHz, D_2O) gave δ: 6.51 (s, 1H, C(6)-H), 4.37 (dd, J=4.8, 3.2 Hz, 1H, C(3)-H), 4.21 (dd, J=8.4, 4.4 Hz, 1H, C(d)-H), 3.75 (d, J=18.0 Hz, 1H, C(f)-H), 3.70 (d, J=18.0 Hz, 1H, C(f)-H), 3.65 (t, J=6.4 Hz, 1H, C(a)-H), 3.21 (dd, J=12.8, 4.8 Hz, 1H, C(2)-H), 3.11 (dd, J=12.8, 3.2 Hz, 1H, C(2)-H), 3.06–2.85 (m, 6H, C(e)-H_2, C(β)-H_2, C(α)-H_2), 2.31 (t, J=7.6 Hz, 2H, C(c)-H_2), 2.00–1.88 (m, 2H, C(b)-H_2).

4.5.7. 6,8-Di-*S*-glutathionyl-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (8-*S*-GS-DHBT-1). This compound was a very pale yellow solid. Dissolved in the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}=330 and 258 nm. In pH 7.4 phosphate buffer λ_{max}, nm (log ϵ_{max}, $M^{-1}\,cm^{-1}$) was 334 (3.52) and 262 (4.35) calculated as 6,8-di-*S*-GS-DHBT-1.2 TFA. FAB-MS (thioglycerol matrix) gave m/z=865.2206 (MH^+, 4%, $C_{31}H_{45}N_8O_{15}S_3$; calcd m/z=865.2167). ^{1}H NMR (400 MHz, D_2O) gave δ: 4.43 (dd, J=4.4, 3.6 Hz, 1H, C(3)-H), 4.24 (dd, J=8.8, 4.8 Hz, 1H, C(d)-H), 4.23 (dd, J=8.8, 4.8 Hz, 1H, C(d′)-H), 3.79 (d, J=18.0 Hz, 1H, C(f)-H), 3.77 (d, J=18.0 Hz, 1H, C(f′)-H), 3.74 (d, J=18.0 Hz, 1H, C(f)-H), 3.72 (t, J=6.4 Hz, 1H, C(a)-H), 3.71 (d, J=18.0 Hz, 1H, C(f′)-H), 3.70 (t, J=6.4 Hz, 1H, C(a′)-H), 3.36–3.24 (m, 2H, C(α)-H_2), 3.21 (dd, J=13.2, 4.4 Hz, 1H, C(2)-H), 3.08 (dd, J=13.2, 3.6 Hz, 1H, C(2)-H), 3.05 (dd, J=14.0, 4.8 Hz, 1H, C(e)-H), 3.04 (dd, J=14.0, 4.8 Hz, 1H, C(e′)-H), 2.98–2.88 (m, 4H, C(e)-H, C(e′)-H, C(β)-H_2), 2.38 (t, J=7.6 Hz, 2H, C(c)-H_2), 2.33 (t, J=7.6 Hz, 2H, C(c′)-H_2), 2.03–1.94 (m, 4H, C(b)-H_2, C(b′)-H_2).

4.5.8. 6-*S*-Glutathionyl-8-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (6-*S*-GS-DHBT-5). This compound was a very pale yellow solid. Dissolved in the HPLC mobile phase (pH 2.15) it showed UV bands at λ_{max}=314, 278 (sh) and 242 nm. In pH 7.4 phosphate buffer the UV spectrum showed λ_{max}, nm (log ϵ_{max}, $M^{-1}\,cm^{-1}$) at 318 (3.46), 280 (sh, 3.83) and 250 (4.33) calculated as 6-*S*-GS-DHBT-5.2 TFA. FAB-MS (thioglycerol matrix) gave m/z=560.1530 (MH^+, 28%, $C_{21}H_{30}N_5O_9S_2$; calcd m/z= 560.1485). ^{1}H NMR (400 MHz, D_2O) gave δ: 6.61 (s, 1H, C(7)-H), 4.46 (dd, J=4.4, 3.6 Hz, 1H, C(3)-H), 4.23 (dd, J=8.8, 4.8 Hz, 1H, C(d)-H), 3.70 (d, J=18.0 Hz, 1H, C(f)-H), 3.70 (t, J=6.4 Hz, 1H, C(a)-H), 3.66 (d, J=18.0 Hz, 1H, C(f)-H), 3.22 (dd, J=13.2, 4.4 Hz, 1H, C(2)-H), 3.11 (dd, J=14.4, 4.8 Hz, 1H, C(e)-H), 3.07–3.02 (m, 3H, C(2), C(α)-H_2), 2.95 (dd, J=14.4, 8.8 Hz, 1H, C(e)-H), 2.74 (t, J= 7.2 Hz, 2H, C(β)-H_2), 2.31 (t, 7.6 Hz, 2H, C(c)-H_2), 2.00–1.94 (m, 2H, C(b)-H_2).

4.5.9. 6,7-Di-*S*-glutathionyl-8-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (6,7-di-*S*-GS-DHBT-5). This compound was isolated as a very pale pink solid. Dissolved in the HPLC mobile phase (pH 2.15) it exhibited UV bands at λ_{max}—326 (sh), 294 (sh) and 262 nm. In pH 7.4 phosphate buffer the UV spectrum showed λ_{max}, nm (log ϵ_{max}, $M^{-1}\,cm^{-1}$) at 332 (sh, 3.60), 302 (sh, 3.76) and 264 (4.34) calculated as 6,7-di-*S*-GS-DHBT-5.2 TFA. FAB-MS (thioglycerol matrix) gave m/z=865.2134 (MH^+, 6%, $C_{31}H_{45}N_8O_{15}S_3$; calcd m/z= 865.2147). ^{1}H NMR (400 MHz, D_2O) gave δ: 4.51 (dd, J=4.4, 3.6 Hz, 1H, C(3)-H), 4.28 (dd, J=8.4, 4.4 Hz, 1H, C(d)-H), 4.10 (dd, J=8.4, 4.4 Hz, 1H, C(d′)-H), 3.74 (t, J=6.4 Hz, 1H, C(a)-H), 3.72 (t, J=6.4 Hz, 1H, C(a′)-H), 3.70 (d, J=18.0 Hz, 1H, C(f)-H), 3.69 (s, 2H, C(f′)-H_2), 3.65 (d, J=18.0 Hz, 1H, C(f)-H), 3.27–3.16 (m, 4H, C(2)-H, C(α)-H_2, C(e′)-H), 3.13 (dd, J=14.4, 4.4 Hz, 1H, C(e)-H), 3.06 (dd, J=14.4, 8.4 Hz, 1H, C(e′)-H), 3.04 (dd, J=14.4, 8.4 Hz, 1H, C(e)-H), 3.00 (dd, J=13.2, 3.6 Hz, 1H, C(2)-H), 2.97–2.91 (m, 2H, C(β)-H_2), 2.38–2.33 (m, 2H, C(c)-H_2), 2.26–2.21 (m, 2H, C(c′)-H_2), 2.03–1.91 (m, 4H, C(b)-H_2, c(b′)-H_2).

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References

1. For recent reviews of Parkinson's disease see: (a) Jenner, P.; Olanow, C. W. Pathological evidence for oxidative stress in Parkinson's disease and related degenerative disorders. In *Neurodegeneration and neuroprotection in Parkinson's disease*; Olanow, C. W., Jenner, P., Youdim, M. B. H., Eds.; Academic: New York, 1996; pp 23–45. (b) Dryhurst, G.; Shen, X.-M.; Li, H.; Han, J.; Yang, Z.; Cheng, F.-C. The chemistry of Parkinson's disease. In *Biomedical chemistry. Applying chemical principles to the understanding and treatment of disease*; Torrence, P. F., Ed.; Wiley-Interscience: New York, 2000; pp 311–346.
2. (a) Schapira, A. H. V.; Mann, V. M.; Cooper, J. N.; Dexter, D.; Daniel, S. E.; Jenner, P.; Clark, J. B.; Marsden, C. D. *J. Neurochem.* **1990**, *55*, 2142–2145. (b) Janetsky, B.; Hauck, S.; Youdim, M. B.; Riederer, P.; Jellinger, K.; Pantucek, F.; Zochling, R.; Boissl, K. W.; Reichmann, H. *Neurosci. Lett.* **1994**, *169*, 126–128.
3. Mizuno, Y.; Matuda, S.; Yoshino, H.; Mori, H.; Hattori, N.; Ikebe, S.-J. *Ann. Neurol.* **1994**, *35*, 204–210.
4. (a) Dexter, D. T.; Carter, C. J.; Wells, F. R.; Javoy-Agid, F.; Agid, Y.; Lees, A.; Jenner, P.; Marsden, C. D. *J. Neurochem.* **1989**, *52*, 381–389. (b) Alam, Z. I.; Daniel, S. E.; Lees, A. J.; Marsden, C. D.; Jenner, P.; Halliwell, B. *J. Neurochem.* **1997**, *69*, 1326–1329. (c) Alam, Z. I.; Jenner, A.; Lees, A. J.; Cairns, N.; Marsden, C. D.; Jenner, P.; Halliwell, B. *J. Neurochem.* **1997**, *69*, 1196–1203. (d) Zhang, J.; Perry, G.; Smith, M. A.; Robertson, D.; Olson, S. J.; Graham, D. G.; Montine, T. J. *Am. J. Pathol.* **1999**, *154*, 1423–1429.
5. (a) Riderer, P.; Sofic, E.; Rausch, W.-D.; Schmidt, B.; Reynolds, G. P.; Jellinger, K.; Youdim, M. B. H. *J. Neurochem.* **1989**, *52*, 515–520. (b) Sian, J.; Dexter, D.; Lees, A.; Daniel, S.; Agid, Y.; Javoy-Agid, F.; Jenner, P.; Marsden, C. D. *Ann. Neurol.* **1994**, *36*, 348–355.
6. (a) Sofic, E.; Riederer, P.; Heisen, H.; Bechmann, H.; Reynolds, G. P.; Habenstreit, G.; Youdim, M. B. H. *J. Neural Transm.* **1988**, *74*, 199–205. (b) Good, P. F.; Olanow, C. W.; Perl, D. P. *Brain Res.* **1992**, *593*, 342–346. (c) Ben-Shachar, D.; Riedere, P.; Youdim, M. B. H. *J. Neurochem.* **1991**, *57*, 1609–1614.
7. (a) Marttila, R. J.; Lorentz, H.; Rinne, U. K. *J. Neurol. Sci.* **1998**, *86*, 321–331. (b) Saggu, H.; Cooksey, J.; Dexter, D.; Wells, F. R.; Lees, A. J.; Jenner, P.; Marsden, C. D. *J. Neurochem.* **1989**, *53*, 692–697.
8. Parboosingh, J. S.; Rousseau, M.; Rogan, F.; Amit, Z.; Chertkow, H.; Johnson, W. G.; Manganaro, F.; Schipper, H. N.; Currau, J. J.; Stoessl, A. J.; Rouleau, G. A. *Arch. Neurol.* **1995**, *52*, 1160–1163.
9. Good, P. F.; Hsu, A.; Werner, P.; Perl, D. P.; Olanow, C. W. *J. Neuropath. Exp. Neurol.* **1998**, *57*, 338–342.
10. Hunot, S.; Boissiere, F.; Faucheux, B.; Brugg, B.; Mouatt-Prigent, A.; Agid, Y.; Hirsch, E. C. *Neuroscience* **1996**, *72*, 355–363.
11. Seaton, T. A.; Jenner, P.; Marsden, C. D. *Biochem. Pharmacol.* **1996**, *52*, 1657–1663.
12. (a) Heales, S. J.; Davies, S. E. C.; Bates, T. E.; Clark, J. B. *Neurochem. Res. 20*, 31–38. (b) Sokol, R. J.; Devereaux, M. W.; O'Brien, K.; Khandwala, R. A.; Loehr, J. P. *Gastroenterology* **1993**, *105*, 178–187. (c) Hartley, A.; Cooper, J. M.; Schapira, A. H. V. *Brain Res. 1993*, *67*, 349–353.
13. (a) Brown, G. C. *Biochim. Biophys. Acta* **1999**, *1411*, 351–369. (b) Lizasoain, I.; Moro, M. A.; Knowles, R. G.; Darley-Usmar, V.; Moncada, S. *Biochem. J.* **1996**, *314*, 877–880.
14. (a) Sharpe, M. A.; Cooper, C. E. *J. Biol. Chem.* **1998**, *273*, 30961–30972; (b) Cassina, A., Radi, R. *Arch. Biochem. Biophys.* **1996**, *328*, 309–316.
15. Mytilineou, C.; Han, S. K.; Cohen, G. *J. Neurochem.* **1993**, *61*, 1470–1478.
16. Pearce, R. K. B.; Owen, A.; Daniel, S.; Jenner, P.; Marsden, C. D. *J. Neural Transm.* **1997**, *104*, 661–677.
17. Dexter, D. T.; Sian, J.; Rose, H.; Hindmarsh, J.-G.; Mann, V. M.; Cooper, J. M.; Wells, F. R.; Daniel, S. E.; Lees, A. J.; Schapira, A. H. V.; Jenner, P.; Marsden, C. D. *Ann. Neurol.* **1994**, *35*, 38–44.
18. Toffa, S.; Kunikowska, G. M.; Zeng, B.-Y.; Jenner, P.; Marsden, C. D. *J. Neural Transm.* **1997**, *104*, 67–75.
19. Sian, J.; Dexter, D. T.; Lees, A. J.; Daniel, S.; Jenner, P.; Marsden, C. D. *Ann. Neurol.* **1994**, *36*, 356–361.
20. (a) Glerlach, M.; Riederer, P. *J. Neural Transm.* **1996**, *103*, 987–1041. (b) Royland, J. E.; Langston, J. W. MPTP: a dopaminergic neurotoxin. In *Highly selective neurotoxins: basica and clinical applications*; Kostrzewa, R. M., Ed.; Humana: Totowa, NJ, 1999; pp 141–194.
21. Han, J.; Cheng, F.-C.; Yang, Z.; Dryhurst, G. *J. Neurochem.* **1999**, *73*, 1683–1695.
22. Ransom, B. R.; Kunis, D. M.; Irwin, I.; Langston, J. W. *Neurosci. Lett.* **1987**, *75*, 323–328.
23. (a) Ramsay, R. R.; Dadgar, J.; Trevor, A.; Singer, T. P. *Life Sci.* **1986**, *39*, 581–588. (b) Cooper, J. M.; Schapira, A. H. V. *J. Bioenerg. Biomembr.* **1997**, *29*, 175–183.
24. Mizuno, Y.; Saitoh, T.; Sone, N. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 971–976.
25. Beal, M. F., *Mitochondrial dysfunction and oxidative damage in neurodegenerative diseases*; R. G. Landes Co.: Austin, TX, 1995.
26. (a) Swerdlow, R. H.; Parks, J. K.; Miller, S. W.; Tuttle, J. B.; Trimmer, P. A.; Scheehan, J. P.; Bennett, J. P.; Davis, R. E.; Parker, W. D. *Ann. Neurol.* **1996**, *40*, 663–671. (b) Kobayashi, T.; Matsumme, H.; Matuda, S.; Mizuno, Y. *Ann. Neurol.* **1998**, *43*, 120–123.
27. (a) Fleming, L.; Mann, J. B.; Briggle, T.; Sanchez-Ramos, J. R. *Ann. Neurol.* **1994**, *36*, 100–103. (b) Sanchez-Ramos, J.; Fucca, A.; Basit, A.; Song, S. *Exp. Neurol.* **1998**, *150*, 263–271. (c) Meco, G.; Bonifati, M. G.; Vanacore, N.; Fabrizio, E. *Scand. J. Environ. Health* **1994**, *20*, 301–305. (d) Bachurin, S. O.; Shevtzova, E. P.; Lermontova, N. N.; Serkova, T. P.; Ramsay, R. R. *Nerurotoxicology* **1996**, *17*, 897–903.
28. (a) Le Couteur, D. G.; McClean, A. J. *Clin. Pharmacokinetics* **1998**, *34*, 359–373. (b) Steventon, G. B.; Heafield, M. T. E.; Waring, R. H.; Williams, A. C. *Neurology* **1989**, *39*, 883–887; Menegon, A.; Board, P. G.; Blackburn, A. C.; Mellick, G. D.; Le Couteur, D. G. *Lancet* **1998**, *352*, 1344–1346. (d) Payami, H.; Zareparsi, S.; *J. Geriatr. Psychiatry Neurol.* **1998**, *11*, 98–106. (e) Bandmann, O.; Vaughan, J.; Holmans, P.; Marsden, C. D.; Wood, N. W. *Lancet* **1997**, *350*, 1136–1139.
29. Rollema, H.; Damsma, G.; Horn, A. S.; De Vries, J. B.; Westerink, B. H. C. *Eur. J. Pharmacol.* **1986**, *126*, 345–346.
30. (a) Zängerle, L.; Cuenod, M.; Winterhalter, K. H.; Do, K.-Q. *J. Neurochem.* **1992**, *59*, 181–189. (b) Wu, E. Y.; Smith,

M. T.; Bellomo, G.; Di Monte, D. *Arch. Biochem. Biophys.* **1990**, *282*, 258–262. (c) Rigobello, M. P.; Bindoli, A. *Mol. Cell. Biochem.* **1993**, *122*, 93–100.
31. (a) Novelli, R.; Reilly, J. A.; Lysko, P. G.; Henneberry, R. C. *Brain Res.* **1988**, *451*, 205–212. (b) Olney, J. W.; Zorumski, C.; Price, M. T.; Labruyere, J. *Science* **1990**, *248*, 596–599.
32. Lafon-Cazal, M.; Pletri, S.; Culcasi, M.; Bockaert, J. *Nature* **1993**, *364*, 535–537.
33. Beal, M. F. Therapeutic effects of nitric oxide synthase inhibition in neuronal injury. In *Neurodegeneration and neuroprotection in Parkinson's disease*; Olanow, C. W., Jenner, P., Youdim, M., Eds.; Academic: New York, 1996; pp 91–101.
34. (a) Flint, D. H.; Tuminello, J. F.; Emptage, M. H. *J. Biol. Chem.* **1993**, *268*, 22369–22376. (b) Brieland, J. K.; Fantone, J. C. *Arch. Biochem. Biophys.* **1991**, *284*, 78–83. (c) Gardner, P. R.; Constantino, G.; Szabo, C.; Salzman, A. L. *J. Biol. Chem.* **1997**, *272*, 25071–25076. (d) Keyer, K.; Imlay, J. A. *J. Biol. Chem.* **1997**, *272*, 27652–27659.
35. Gutteridge, J. M. C. *Ann. N.Y. Acad. Sci.* **1996**, *738*, 201–213.
36. Crow, J. P.; Spruell, C.; Chen, J.; Gunn, C.; Ischiropoulos, H.; Tsai, M.; Smith, C. D.; Radi, R.; Koppenol, W. H.; Beckman, J. S. *Free Rad. Biol. Med.* **1994**, *16*, 331–338.
37. (a) Smith, T. S.; Bennett, J. P. *Brain Res.* **1997**, *765*, 183–188. (b) Chiueh, C. C.; Wu, R.-M.; Mohanakumar, K. P.; Sternberger, L. M.; Krishna, G.; Obtata, T.; Murphy, D. L. *Ann. N.Y. Acad. Sci.* **1994**, *738*, 25–36. (c) Giovanni, A.; Liang, L. P.; Hastings, T. G.; Zigmond, M. J. *J. Neurochem.* **1995**, *64*, 1819–1825. (d) Althaus, J. S.; Andrus, P. K.; Williams, C. M.; Von Voigtlander, P. F.; Cazars, A. R.; Hall, E. D. *Mol. Chem. Neuropath.* **1993**, *20*, 147–162. (e) Thomas, B.; Muralikrishnan, D.; Mohanakumar, K. P. *Brain Res.* **2000**, *852*, 221–224.
38. (a) Cooper, A. J. L. Role of astrocytes in maintaining cerebral glutathione homeostasis and protecting the brain against xenobiotics and oxidative stress. In *Glutathione in the nervous system*; Shaw, C. A., Ed.; Taylor & Francis: Washington, DC, 1998; pp 91–115. (b) Dringen, R.; Kranich, O.; Hamprecht, B. *Neurochem. Res.* **1997**, *22*, 727–733.
39. Spencer, J. P. E.; Jenner, P.; Daniel, S. E.; Lees, A. J.; Marsden, C. D.; Halliwell, B. *J. Neurochem.* **1998**, *71*, 2112–2122.
40. (a) Kerry, N.; Rice-Evans, C. *J. Neurochem.* **1999**, *73*, 247–253. (b) Daveu, C.; Servy, C.; Dendane, M.; Marin, P.; Ducrocq, C. *Nitric Oxide* **1997**, *1*, 234–243.
41. Tate, S. S.; Meister, A. A. *Methods Enzymol.* **1985**, *113*, 400–437.
42. (a) Sagara, J.; Miura, K.; Bannai, S. *J. Neurochem.* **1993**, *61*, 172–1676. (b) Dringen, R.; Pfeiffer, B.; Hamprecht, B. *J. Neurosci.* **1999**, *19*, 562–569.
43. Inoue, M.; Saito, Y.; Hirata, E.; Morito, Y.; Nagase, S. *J. Protein Chem.* **1987**, *6*, 207–225.
44. Carboni, S.; Melis, F.; Pani, L.; Hadjiconstantinou, M.; Rossetti, Z. K. *Neurosci. Lett.* **1990**, *117*, 129–133.
45. (a) Zhang, F.; Dryhurst, G. *J. Med. Chem.* **1994**, *37*, 1084–1098. (b) Shen, X.-M.; Dryhurst, G. *Chem. Res. Toxicol.* **1996**, *9*, 751–763. (c) Zhang, F.; Dryhurst, G. *Bioorg. Chem.* **1995**, *23*, 193–216.
46. Fornstedt, B.; Brun, A.; Rosengren, E.; Carlsson, A. *J. Neural Transm. [Parkinson's Dis. Dement. Sect.]* **1989**, *1*, 279–295.
47. (a) Li, H.; Dryhurst, G. *J. Neurochem.* **1997**, *69*, 1530–1541. (b) Li, H.; Shen, X.-M.; Dryhurst, G. *J. Neurochem.* **1998**, *71*, 2049–2062; Shen, X.-M.; Li, H.; Dryhurst, G. *J. Neural Transm.* **2000**, in press.
48. Weiner, H. L.; Hashim, A.; Lajtha, A.; Sershen, H. *Res. Commun. Subst. Abuse* **1988**, *9*, 53–68; Osihi, T.; Hasegawa, E.; Murai, Y. *J. Neural Transm. [Parkinson's Dis. Dement. Sect.]* **1993**, *6*, 45-52.
49. Shen, X.-M.; Zhang, F.; Dryhurst, G. *Chem. Res. Toxicol.* **1997**, *10*, 147–155.
50. Zhang, F.; Dryhurst, G. *J. Electroanal. Chem.* **1995**, *398*, 117–128.
51. Sriram, K.; Pai, K. S.; Boyd, M. R.; Ravindranath, V. *Brain Res.* **1997**, *749*, 44–52.
52. (a) Steranka, L. R.; Rhina, A. W. *Eur. J. Pharmacol.* **1987**, *133*, 191–197. (b) Yamamoto, M.; Sakamoto, N.; Iwai, A.; Yatsugi, S.; Hidaka, K.; Noguchi, K.; Yuasa, T. *Res. Commun. Chem. Pathol. Pharmacol.* **1993**, *81*, 221–232.
53. Sechi, G.; Deledda, M. G.; Bua, G.; Satta, W. M.; Dejana, G. A.; Pes, G. M.; Rosati, G. *Prog. Neuro-psychopharmacol. Biol. Psychiatry* **1996**, *20*, 1159–1170.
54. Andén, N.-E.; Fuxe, K.; Hamberger, B.; Hökfelt, T. *Acta Physiol. Scand.* **1966**, *67*, 306–312.
55. (a) Kandel, E. R., Schwartsz, J. H., Jessell, T. M., Eds. *Principles of Neural Science*; 3rd ed.; Elsevier: New York, 1991. (b) Hochstetler, S. E.; Puopolo, M.; Gustincich, S.; Raviola, E.; Wightman, R. M. *Anal. Chem.* **2000**, *72*, 489–496.
56. Rice, M. E.; Russo-Menna, I. *Neuroscience* **1998**, *82*, 1213–1223.