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Intraneuronal Dopamine–Quinone Synthesis: A Review

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Dopamine–quinone is synthesized by oxidation of the catechol ring of dopamine. If this occurs within the neuronal cytosol, the quinone may react with cytosolic components, particularly cysteine residues. In contrast, if quinone is produced within neuronal lysosomes it may provide the fundamental building block for neuromelanin. Since the population of neurons that die in Parkinson's disease are those that display obvious intralysosomal neuromelanin and since cytosolic dopamine-dependent oxyradical formation may underlie methamphetamine toxicity and other specific forms of neurodegeneration in dopaminergic neurons, it is important to elucidate the pathways leading to dopamine–quinone. Here we review pathways by which intracellular catechols may be oxidized to quinones, either enzymatically or via reduction of ferric iron or other metals. These metabolites can be adduced by cysteine, could underlie aberrant metabolism and ubiquitination pathways, may induce Lewy body formation, and mediate the synthesis of hydroxyl radical and oxyradical species. Finally, we suggest that by accumulating excess cytosolic catecholamine, neuromelanin synthesis may safely sequester quinones that would otherwise be produced in neuronal cytosol.

Keywords: Neuromelanin, Parkinson's disease, Lewy bodies, Methamphetamine, Oxidative stress, VMAT, Substantia nigra, Tyrosinase

Abbreviations: AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; DA, dopamine; DAT, dopamine transporter (plasma membrane); DHBT, dihydrobenzothiazine; DLBD, diffuse Lewy body disease; DOPAC, dihydroxyphenylacetic acid; GDNF, glial-derived neurotrophic factor; HPETE, arachidonic acid hydroperoxide; LBs, Lewy bodies; L-NAC, L-n-acetylcysteine; L-DOPA, L-3,4-dihydroxyphenylalanine; mRNA, messenger RNA; METH, methamphetamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NE, norepinephrine; NM, neuromelanin; PD, Parkinson's Disease; PKC, protein kinase C; RT-PCR, reverse transcription polymerase chain reaction; SN, substantia nigra; SNC, substantia nigra pars compacta; SOD, superoxide dismutase; TH, tyrosine hydroxylase; VMAT1, peripheral vesicular monoamine transporter; VMAT2, central vesicular monoamine transporter; VTA, ventral tegmental area; 6-OHDA, 6-hydroxydopamine

INTRODUCTION

Parkinson's disease (PD) is characterized by progressive degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNC) and norepinephrine (NE) neurons of the locus coeruleus (LC) (Bertrand *et al.*, 1997), and lesser

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degeneration of DA neurons in the ventral tegmental area (VTA) (McRitchie *et al.*, 1997). The most prominent distinguishing characteristics of these neurons are that they express high levels of intracellular neuromelanin (NM), and in the case of PD, intracellular ubiquitinated inclusions known as Lewy bodies (LBs). While the cause of idiopathic PD is unidentified, there is considerable evidence associating the disorder with heightened oxidative stress (Fahn and Cohen, 1992; Jenner and Olanow, 1996). In particular, there are increased levels of lipid peroxidation, iron levels, and elevated superoxide dismutase (SOD) activity in PD brains (Hirsch *et al.*, 1992).

The mechanism underlying this increased oxidative stress may involve DA itself. In neuronal culture, 100 μM DA applied extracellularly is highly neurotoxic, a response blocked by catalase (Rosenberg, 1988; Michel and Hefti, 1990). Furthermore, antioxidants or neurotrophic factors confer protection against DA or DA metabolite-induced degeneration. For example, SOD overexpression protects against methamphetamine (METH) and L-3,4-dihydroxyphenylalanine (L-DOPA)-induced neurotoxicity in transgenic mice (Przedborski *et al.*, 1992; Cadet *et al.*, 1994; Mena *et al.*, 1997), while brain-derived neurotrophic factor (BDNF) via elevation of glutathione protects mesencephalic cultures from 6-hydroxyDA (6-OHDA) neurotoxicity (Spina *et al.*, 1992).

Neurodegeneration by High Cytosolic Dopamine is Suggested by Methamphetamine Studies

Studies of METH neurotoxicity introduced the hypothesis that oxidation of cytosolic DA may be deleterious to neurons. METH evokes an unusual pattern of neurotoxicity in which DA axonal processes degenerate while cell bodies are left intact (Seiden *et al.*, 1993). METH neurotoxicity in postnatal midbrain cultures resembles METH-induced neurodegeneration *in vivo* in several ways (Cubells *et al.*, 1994). There is little loss of tyrosine hydroxylase (TH)-positive cell bodies in culture, although most TH-labeled processes develop blebs and swollen varicosities and degenerate within one week.

Intracellular DA can be localized in a number of intracellular pools (Fig. 1). It appears that DA-dependent toxicity in the neuronal cultures results from the redistribution of vesicular DA from the synaptic vesicle, where it is maintained in a reduced state, to the cytosol where it can undergo oxidation. This is due to the ability of synaptic vesicle to accumulate amphetamine or METH. These drugs are weak bases that bind intravesicular protons and collapse the pH gradient that provides the energy for transmitter accumulation in the vesicle (Sulzer and Rayport, 1990; Sulzer *et al.*, 1995). Synaptic vesicles presumably maintain catecholamines in a reduced state, due to the low pH within the vesicle. The redistribution of DA from vesicles to the cytosol would be expected to produce local sites of cytosolic oxidative stress after the oxidative buffering capacity of the cytosol is exceeded. This model of METH neurotoxicity, which was deduced from experiments in neuronal culture, was recently confirmed in two *in vivo* studies. Heterozygous (+/−) vesicular monoamine transporter-2 (VMAT2) mutant mice that display only ~50% of normal DA content were found to be far more susceptible to METH toxicity (Fumagalli *et al.*, 1999). Nevertheless, +/− mice showed less METH-evoked DA release and less extracellular oxyradical formation than wild-type littermates. Therefore, the authors suggest that cytosolic rather than extracellular DA oxidation is primarily responsible for METH-evoked neurotoxicity. In an independent study, increased cytosolic DA–quinone synthesis was implicated following METH exposure (LaVoie and Hastings, 1999) by the increased presence of DA–cysteinyl adducts that are downstream metabolites of DA–quinones (see below).

In summary, results from METH experiments suggest that high levels of cytosolic DA induce oxidative stress and neurodegeneration. Since catecholamine oxidation occurs by conversion to

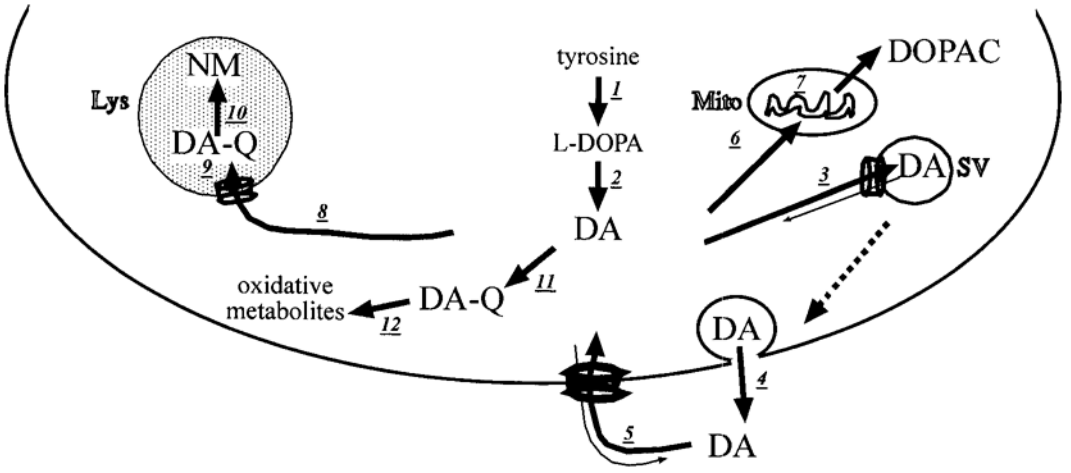

FIGURE 1 Fates for intraneuronal DA. *Traditional view of DA metabolism, steps* 1–7. (1) Free cytosolic tyrosine hydroxylase converts tyrosine to L-DOPA. (2) Free cytosolic aromatic decarboxylase converts L-DOPA to DA. (3) The vesicular monoamine transporter (VMAT2) mediates synaptic vesicle sequestration of cytosolic DA. DA can also be released into the cytosol via reverse transport through VMAT2, particularly following amphetamine or reserpine (thin arrow). (4) Following translocation of the vesicle to a docking site on the plasma membrane (broken arrow), DA is released from the neuron by vesicle exocytosis. (5) The DA uptake transporter (DAT) accumulates extracellular DA into the cytosol. Under some conditions, particularly following amphetamine, DA is released via reverse transport through the DAT (thin arrow). (6) Cytosolic DA permeates the mitochondrial membrane. (7) Mitochondrial monoamine oxidase reacts with DA to produce DOPAC. *DA metabolism via quinone reactions, steps* 8–12. (8) DA appears to be sequestered in lysosomes and related secretory organelles by VMAT2. (9) Within lysosomes, DA is converted enzymatically or via a metal catalysis to DA-quinone. (10) DA–quinone is polymerized to NM, which is sequestered in the lysosome away from the cytosol. It may be that pigmented nigral neurons result from a lifetime of such protection. (11) If not buffered by the pathways indicated above, cytosolic DA can be oxidized to DA–quinone, a reaction that also promotes formation of small oxyradicals. (12) DA–quinone reacts with cysteine and other macromolecular targets to that can induce neuronal toxicity.

a quinone, we review steps by which intracellular oxidation of DA could occur. An alternate oxidation pathway that will not be discussed is conversion to 6-OHDA (Napolitano *et al.*, 1995; Pezzella *et al.*, 1997), a highly reactive DA metabolite capable of removing iron from ferritin *in vitro* (Youdim and Riederer, 1993); however, many reactions discussed may pertain to this metabolite as well.

MECHANISMS OF QUINONE FORMATION

Stoichiometry of Electrons Donated by Dopamine Autooxidation

Oxidation of catecholamines can occur by two sets of reactions (Graham, 1978; Ciolkowski *et al.*, 1994); see also the recent review by Stokes *et al.* (1999). The reactions that donate electrons are shown as Reactions 1 and 3 in Fig. 2. In Reaction 1, conversion of the catecholamine to a catecholamine-*o*-quinone, occurs rapidly and donates $2e^-$ to an electron acceptor. If only one of the hydroxyls is oxidized, the compound is a *semi*-quinone. In Reaction 2, the amine can either be deprotonated and undergo cyclization to leucoaminochrome or be re-reduced to the catecholamine. In Reaction 3, the oxidation of leucoaminochrome to aminochrome results in the donation of 2 additional e^- to an electron acceptor. Under conditions of restricted diffusion, as when catecholamines are trapped in a water droplet surrounded by oil, the current following oxidation by application of +800 mV using a carbon fiber microelectrode is well fit by $4e^-$ per molecule (Bruns and Jahn, 1995).

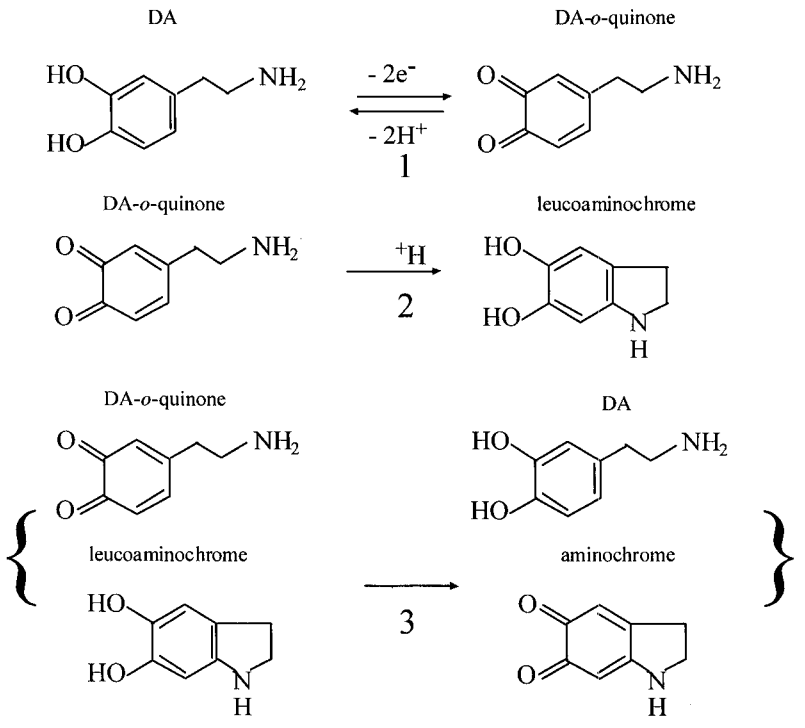

FIGURE 2 Oxidative reactions for DA. Reaction 1 is a rapid electrochemical reaction favored at higher pH that donates $2e^-$. Reaction 2 is a cyclization favored at lower pH. Reaction 2 is generally rate-determining for the donation of the second set of $2e^-$, because leucoaminochrome is efficiently converted to aminochrome in Reaction 3, where it donates an additional $2e^-$. In this step, the leucoaminochrome together with a quinone regenerates DA as well (a *disproportionation* reaction), so that the reaction also consumes $2e^-$. As can be seen, with sufficient oxidation potential and sufficient H^+ for the second reaction, $4e^-$ would eventually be donated by each DA molecule in its conversion to aminochrome (Ciolkowski *et al.*, 1994).

Reaction 1 is strongly favored at alkaline pH. Cyclic voltammetry traces indicate that at a pH of 7.4 in physiological saline, the oxidation peak for DA is ~ 300 mV. As the pH becomes more acidic, the voltage at which peak oxidation occurs is increased by $\sim +50$ mV per unit.

In contrast, Reaction 2 is much slower and is favored at acidic pH. The cyclization is via an internal Michael addition, which forms leucodopaminochrome (indoline-5,6-diol). The cyclization is strongly favored at acidic pH but is quite slow at physiological pH. The reason for the slow rate is thought to be since for cyclization to proceed, two intermediate states must occur, (1) the amino group must be deprotonated (pK = 9.9), which is favorable at alkaline pH, *and* (2) the quinone must be protonated, which is favorable at acidic pH.

The rate of oxidation at Reaction 3 is limited by the rate of Reaction 2. In a study by Wightman's group (Ciolkowski *et al.*, 1994), the rate constant for formation of the second set of electrons from DA via Reaction 3 at physiological pH when +700 mV was applied from a carbon fiber electrode was $0.07 \pm 0.03\ s^{-1}$, i.e., a time constant τ of $1/0.07\ s = 14.3\ s$.

Typical resting potentials are ~ -50 to -70 mV. While an action potential would provide a short-lived depolarization to $\sim +50$ mV for < 1 ms, this would be insufficient to oxidize a significant fraction of cytosolic DA by Reaction 1. Given the high potentials that would need to be applied at

relevant pH, it might appear that catecholamines should remain stable in the cytosol.

The Role of Metals and Oxygen Free Radicals in the Synthesis of DA–Quinone

However, this situation is very different in the presence of metals that promote oxidation, such as ferric iron (Fe^{3+}). Under these conditions, Reaction 1 is mediated by a short-lived complex with the hydroxyl groups bound to Fe^{3+}. The metal donates an electron to produce a short-lived semiquinone and free Fe^{2+}. This is followed by another Fe^{3+} to Fe^{2+} reduction to produce DA–quinone. In the presence of Fe^{3+}, the one-electron redox potential for semiquinone is only +18 mV, and so the semiquinone is rapidly converted to the quinone. Moreover, in the presence of Fe^{3+}, the rate of Reaction 1 is favored even at pH 0.78 (El-Ayaan *et al.*, 1997). The rate of formation of the iron complex is even more favored at higher pH since $Fe(OH)^{2+}$ is more reactive than Fe^{3+} itself.

Similarly, Reaction 3 proceeds via reduction of $2Fe^{3+}$. At $pH < 3$, DA–quinone levels can be followed by its absorbance and the iron–DA complex followed due to its green pigment. At $pH > 3$, where there is absorbance from Fe^{3+} complexes, rates can be estimated using periodate as an oxidant. In this case, the estimated rate constant for Reaction 2 at physiological pH is $\sim 0.1/s$ and the time constant τ would be $\sim 10\,s$ (El-Ayaan *et al.*, 1997), which would provide a long delay prior to Reaction 3. Similar results have been demonstrated for norepinephrine oxidation (El-Ayaan *et al.*, 1998).

What happens to the electrons generated during these reactions? When using a carbon fiber electrode in cyclic voltammetry, the charge is carried through the electrode as current. In the case of metal cofactors, the metals are reduced. Reduced iron via the Fenton reaction or Haber Weiss reaction (in the presence of hydrogen peroxide) generates extremely reactive hydroxyl radicals. Other metals such as copper and manganese (Lloyd, 1995) can mediate quinone formation from DA and increase the rate of hydrogen peroxide and oxyradical production during quinone synthesis (Graham, 1984; Marinho and Manso, 1993; Montine *et al.*, 1995).

Moreover, other compounds are capable of nucleophilic attack. The spontaneous oxidation of catecholamines in the presence of molecular oxygen *in vitro* produces hydrogen peroxide, hydroxyl radical, and superoxide (Graham, 1978; Klegeris *et al.*, 1995; Marinho and Manso, 1993). Therefore, it seems that not only the quinone product, but small radicals could elicit neurodegenerative actions following synthesis of DA–quinone (Fahn and Cohen, 1992). Nitric oxide can also react with DA, producing a 6-nitro derivative (Daveu *et al.*, 1997). In contrast, peroxynitrite produces oxidized DA derivatives (Daveu *et al.*, 1997; LaVoie and Hastings, 1999).

Interestingly, while DA and L-DOPA increase production of hydroxyl radical via the Fenton reaction, their O-methylated derivatives, which are produced by catechol-O-methyl transferase, protect against formation of hydroxyl radicals (Miller *et al.*, 1996; Nappi and Vass, 1998).

Enzymes Involved in DA–Quinone Synthesis

In the 1920's, Raper suggested that the so-called eumelanins that comprise the pigment of hair and skin were comprised of a regular polymer of indole-5,6-quinone, and that the enzyme tyrosinase (*a.k.a.* monophenol monooxygenase) catalyzes both the conversion of tyrosine to L-DOPA and then L-DOPA to dopa-quinone (Pearse, 1985; Sanchez-Ferrer *et al.*, 1995) (Fig. 3). Recently, tyrosinase mRNA was shown to be present in SN neurons using RT-PCR (Xu *et al.*, 1997) and a beta-galactosidase reporter gene to monitor tyrosinase promoter activity *in vivo* (Tief *et al.*, 1998). Therefore, L-DOPA or DA may be enzymatically converted to a quinone in these neurons. The subcellular location, if any, of tyrosinase protein in the SN is currently unknown and at this time

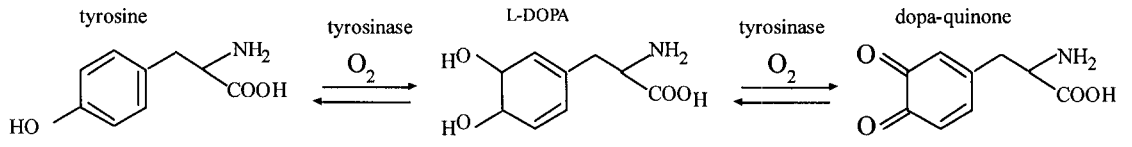

FIGURE 3 Proposed scheme of melanogenesis for eumelanins as proposed by Raper as restated by Pearse (Pearse, 1985). In this case, L-DOPA is the substrate as it is in melanocytes, but tyrosinase also produces quinones from DA (Korytowski *et al.*, 1987), and if this occurs in NM formation, the substrate would likely be DA packaged in lysosomes by VMAT.

the role of this enzyme in central DA neurons remains unclear.

Additional enzymes may be involved in quinone synthesis. Prostaglandin H synthase (a.k.a. cyclooxygenase) has two distinct catalytic activities, a cyclooxygenase and a peroxidase. During conversion of arachidonic acid to prostaglandin H, this enzyme can use DA as a cofactor and cause conversion to dopaminochrome. It can also use hydrogen peroxide as a substrate for quinone conversion (Hastings, 1995; Mattammal *et al.*, 1995). This enzyme is activated by phospholipase A2-mediated arachidonic acid release, and so a variety of protein kinase C (PKC)-mediated pathways could alter cytosolic DA oxidation. Arachidonic acid hydroperoxide (HPETE) in the presence of iron also promotes DA oxidation and formation of DA cysteine adducts (Palumbo *et al.*, 1995). Other enzymes that could be involved include lipoxygenase (Blarzino *et al.*, 1999; Mosca *et al.*, 1996; Rosei *et al.*, 1994), cytochrome C (Rosei *et al.*, 1998a), ceruloplasmin (Rosei *et al.*, 1998b), xanthine oxidase (Foppoli *et al.*, 1997), monoamine oxidase, and peroxidase (d'Ischia and Prota, 1997). However, it remains difficult to estimate the extent by which enzymes mediate these reactions.

L-DOPA has been identified as a potential substrate for TH. In this case, TH produces L-DOPA–quinone (Haavik, 1997). In the presence of cysteine and other groups, this DOPA oxidase activity of TH produces thioether derivatives (Haavik, 1997), which may include cysteine adducts. A variant form of catechol autooxidation in the presence of aldehydes can produce quinone derivatives that have been found in PD brain (Mosca *et al.*, 1998).

Cysteine Adducts

DA–quinone would crosslink proteins via cysteine residues, as has been shown for neurofilament protein (Montine *et al.*, 1995) and as suggested in production of DNA adducts (Stokes *et al.*, 1996). 5-S-cysteinyl–DA has been found in human SN (Rosengren *et al.*, 1985), and its presence, along with similar 5-S-cysteinyl adducts of DOPA and DOPAC, demonstrated that quinone-forming pathways must exist in the brain (Fornstedt *et al.*, 1989). The formation of these adducts is often likely via glutathione derivatives, as there is a higher concentration of glutathione with respect to cysteine (Ratan *et al.*, 1996). Proteases may then convert the glutathione derivatives to cysteine derivatives.

Quinone adducts to exposed cysteine residues in proteins could provide the basis for protein adducts following DA exposure (Hastings and Zigmond, 1994; Ito *et al.*, 1988; LaVoie and Hastings, 1999). As cysteine is generally an important residue for protein function, DA–quinone adducts to cysteine would tend to destroy normal function (Fig. 4).

5-S-cysteinyl–DA itself undergoes further oxidation to produce several dihydrobenzothiazines derivatives. Both 5-S-cysteinyl–DA and these downstream metabolites are more easily oxidized than DA itself, and have been observed to be highly neurotoxic following injection into mouse brain (Zhang and Dryhurst, 1994).

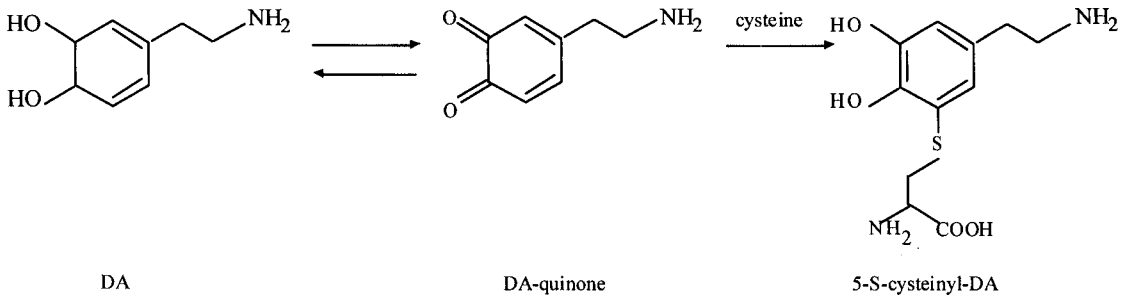

FIGURE 4 Cysteine can react with the DA–quinone ring to produce 5-S-cysteinyl–DA. This could provide a nucleus by which a DA metabolite could react with proteins.

5-S-cysteinyl–DA has been suggested to be a major building block of NM (Carstam *et al.*, 1991; Smythies, 1996). This would suggest that DA–quinone synthesis and adduction by cysteine occurs in lysosomes as well as cytosol (see below). However, one study (Wakamatsu *et al.*, 1991) suggests that cysteine does not actually participate in the formation of NM. At present, several precursors and pathways (enzymatic or not) can be proposed for NM synthesis, but there are no data showing that one reaction is specifically involved.

Recent studies have examined the synergistic reactions between Fe^{3+} and Mn^{2+} and cysteine on DA autoxidation (Palumbo *et al.*, 1995; Shen *et al.*, 1997; Shen and Dryhurst, 1998). Fe^{2+}/Fe^{3+} and cysteine together generate hydroxyl and cysteinyl radicals. DA semiquinone or quinone then reacts with cysteine to give 5-S-cysteinyl–DA as the major product and 2-S-cysteinyl–DA and 6-S-cysteinyl–DA as the minor metabolites. 5-S-cysteinyl–DA can be further oxidized by hydroxyl radical to an *o*-quinone that cyclizes to an *o*-quinone–imine, a precursor of dihydrobenzothiazine (DHBT) and other cyclized products. Cysteinyl conjugates of DA also produce DA thiyl radicals and a variety of DA disulfides and thioethers.

The Mn^{2+}-catalyzed oxidation of DA generates DA–quinone that is scavenged by cysteine to generate 5-S-cysteinyl–DA with lower yields of other isomers of cysteinyl–DA. Subsequent Mn^{2+}-catalyzed oxidation of 5-S-cysteinyl–DA produces DHBTs.

SUGGESTED CELLULAR RESPONSES TO INTRANEURONAL QUINONE

Quinones may Promote Protein Ubiquitination and Initiate Lewy Body Formation

Abnormally upregulated ubiquitination of cytosolic proteins has been suggested to precede and/or initiate the formation of LBs in the cytosol of DA neurons in PD. Importantly for PD studies, ubiquitin appears to be responsible for degradation of proteins damaged by oxyradicals (Conconi and Friguet, 1997; Grune *et al.*, 1997). Ubiquitination is promoted by hydrophobic amino acid residues, aromatic residues, and bulky aliphatic residues exposed during the oxidative rearrangement of protein structure; indeed, increased surface hydrophobicity is a feature common to all oxidized proteins. The recognition of such normally shielded ubiquitinated hydrophobic residues has been suggested to mark oxidatively modified proteins for digestion by the proteasome (Baumeister and Lupas, 1997). By minimizing protein aggregation and cross-linking and removing potentially toxic protein fragments, the proteasome plays a key role in the overall antioxidant defenses. Cadmium, a heavy metal known to induce oxidative stress, promotes

ubiquitination of total cell proteins in midbrain neuronal cultures (Figueiredo-Pereira *et al.*, 1997). Significantly, inhibition of proteasome function in yeast and mammalian cells, presumably due to accumulation of unfolded proteins, triggers the expression of heat shock proteins and increases cell resistance to high temperature and various toxic insults (Goldberg *et al.*, 1997).

It is possible that direct DA–quinone binding to cysteine or quinone-derived oxyradicals that react with proteins initiate these pathways. While LBs have a broad variety of protein constituents (see Table I), neurofilaments are most obvious at the ultrastructural level. A study of neurofilament degradation in a cell-free system demonstrated that addition of ATP and exogenous ubiquitin stimulated proteolysis of neurofilament by crude fractions of cytosolic enzymes (Gou and Leterrier, 1995), proving that ubiquitin can mediate neurofilament degeneration. In a cell-free system, DA, L-DOPA, or DOPAC crosslinks neurofilaments, indicating that quinone products may form the neurofilament LB core. Moreover, this crosslinking mechanism is promoted by copper, iron, or manganese ions (Montine *et al.*, 1995). Interestingly, in this experiment it was shown that L-N-acetylcysteine (L-NAC), a sulfhydryl reducing agent and glutathione precursor, blocks neurofilament crosslinking. While this approach is limited in that it cannot form genuine cellular LB-like organelles, it does indicate a potential role for the reaction of DA–quinone and proteins in LB development.

It has been suggested that an initial step in LB formation may be induced by the conversion of proteins into incorrectly folded beta sheets that form a nucleus for LB formation, possibly following mediation by molecular chaperones, such as heat shock proteins (Welch and Gambetti, 1998). The proteins located in LBs (Table I) are prominent candidates for proteins that may be modified by DA–quinones. One of these protein targets could be alpha-synuclein, which in two mutant forms causes familial PD (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997) and which is

TABLE I Protein constituents of LB identified by immunohistochemistry. DLBD, diffuse Lewy body disease; PD, Parkinson's disease; PLC, phospholipase C

Filaments
MAP 5 (more than MAP 2) (Gai *et al.*, 1996)
Neurofilament subunit proteins H, M, L (200, 160, 70 mw) (Bancher *et al.*, 1989; Fukuda *et al.*, 1993) (Goldman *et al.*, 1983; Galvin *et al.*, 1997)
Neurofilament H and M (in DLBD) (Pollanen *et al.*, 1993)
Proteasome/ubiquitin related
MS73 (a regulatory subunit of proteasome 26S) (Fergusson *et al.*, 1996)
20 S (the catalytic core, and a portion of the proteasome 26S complex) (It *et al.*, 1997)
Proteasome antigens (in DLBD) (Masaki *et al.*, 1994)
Ubiquitinated proteins (actin-like) (Mather *et al.*, 1993) (neurofilament-like in DLBD) (Bancher *et al.*, 1989)
Ubiquitin (in DLBD) (Masaki *et al.*, 1994)
Synaptic/secretory vesicle-related proteins
Alpha-synuclein (PD LB) (Spillantini *et al.*, 1997)
Alpha-synuclein (DLBD LB) (Takeda *et al.*, 1998)
Chromogranin A (Nishimura *et al.*, 1994)
Synaptophysin (Nishimura *et al.*, 1994) (Takeda *et al.*, 1998)
Not determined at this writing
Parkin (Kitada *et al.*, 1998)
Cytosolic proteins
Alpha-B-crystallin (Lowe *et al.*, 1993)
Calbindin-D 28K (Fukuda *et al.*, 1993)
Calpain II (M-calpain) (Mouatt-Prigent *et al.*, 1996)
Cdk5 (Brion and Couck, 1995)
Copper zinc superoxide dismutase (SOD1) (Nishiyama *et al.*, 1995)
Cytoplasmic dynein (Nishiyama *et al.*, 1995)
Heme oxygenase 1 (Castellani *et al.*, 1996)
MxA protein (Yamada, 1995)
p35nck5a (Nakamura *et al.*, 1997)
Tyrosine hydroxylase (midbrain neurons in PD) (Nakashima and Ikuta, 1984)
Tyrosine hydroxylase (catecholaminergic medullary neurons in PD) (Kato *et al.*, 1995)
In DLBD but absent in PD LB
Phospholipase C (PLC) isozyme, PLC delta (Shimohama *et al.*, 1993)
Tau protein (Bancher *et al.*, 1989) (Fukuda *et al.*, 1993)
Tropomyosin (Fukuda *et al.*, 1993)
Identified in DLBD, not reported for PD
Ubiquitin carboxyl terminal hydrolase (PGP 9.5) (Lowe *et al.*, 1990)
Absent in LB
Alpha-interferon (Yamada, 1995)
Beta-amyloid (Murphy *et al.*, 1994)
Glial fibrillary protein (Fukuda *et al.*, 1993)
Myelin basic protein (Fukuda *et al.*, 1993)

present in LBs (Baba *et al.*, 1998; Spillantini *et al.*, 1997; Wakabayashi *et al.*, 1997). It has also been suggested that a central *pale* non-filamentous area may precede full LB formation (Hayashida *et al.*, 1993); these pale bodies are immunopositive for α-synuclein (Irizarry *et al.*, 1998). Additional protein targets, including DAT, glutamate transporters, and tyrosine hydroxylase (Stokes *et al.*, 1999) and serotonin binding protein (Velez-Pardo *et al.*, 1998), have been suggested as potential sites of quinone modification, as has DNA (Stokes *et al.*, 1999).

One new reason to suspect involvement of the ubiquitin pathway in PD is because the *parkin* gene that appears to underlie autosomal recessive juvenile parkinsonism matches ubiquitin in its first 72 amino acids (~33% identity, ~66% similarity). However, the causal mechanisms may be different since this particular disease does not display LBs (Kitada *et al.*, 1998).

An additional ubiquitin-associated mutation has been identified that underlies a familial PD variant, a missense mutation (Ile93Met) in *ubiquitin carboxy-terminal hydrolase L1* (UCH-L1, a.k.a. PGP 9.5) gene. This hydrolase, which represents as much as 2% of total brain protein, is a deubiquitinating enzyme thought to hydrolyze bonds between ubiquitin and small molecules (Larsen *et al.*, 1996). UCH-L1 has been located in LBs in diffuse Lewy body disease (DLBD) (Lowe *et al.*, 1990), but its presence has not yet been reported in PD LBs.

In Alzheimer's disease (AD) and Down's patients, although not PD patients, ubiquitin-B can undergo a frame shift mutation due to a dinucleotide deletion from GAGAG motifs that results in an aberrant COOH-terminus (van Leeuwen *et al.*, 1998), suggesting a direct role played by aberrant ubiquitin in some disorders. Ubiquitin itself is strongly present in LBs and other types of neuronal inclusions, including those that contain amyloid (Mayer *et al.*, 1996) and alpha-synuclein. Interestingly, amyloid beta protein (Abeta), a component of plaques and tangles in AD, has been shown to inhibit proteasome activity, suggesting the possibility that at least these ubiquitinated inclusion bodies may be formed due to blockade of proteasomal-mediated degradation (Gregori *et al.*, 1995).

Quinones may Interfere with Mitochondrial Function

Another target of quinone damage could be at the mitochondria. DA can inhibit NADH reductase activity in isolated rat mitochondria. NE can also cause such an inhibition but it is less potent than DA (Ben-Shachar *et al.*, 1995). DA derivatives such as DHBT that are formed via oxidation of 5-S-cysteinylDA can irreversibly block NADH reductase in isolated mitochondria (Li and Dryhurst, 1997); however it is not yet demonstrated that DHBT is present in the SN. Clearly, numerous other enzymes, particularly with exposed cysteines, could be targets of quinone attack.

Mitochondrial toxicity mediated by quinones has been suggested to produce apoptosis in PD (Merad-Boudia *et al.*, 1998). DA induces apoptosis in PC12 cells and other culture systems (Stokes *et al.*, 1999; Walkinshaw and Waters, 1995), particularly in the presence of Fe^{2+} (Velez-Pardo *et al.*, 1997), although the role of apoptosis in PD remains controversial. DA-induced apoptosis may be due to intraneuronal DA–quinones, since apoptosis was diminished by blockade of the DA transporter (Simantov *et al.*, 1996).

Quinone Synthesis in Lysosomes may Initiate Neuromelanin Formation

Given the seemingly favorable conditions for cytosolic DA–quinone synthesis and the threat that this substance poses, it would seem that cytosolic levels of DA must be maintained at low levels for the health of the neuron. Protection against low levels of DA–quinone would be provided by endogenous antioxidants such as glutathione that could block cytosolic DA metabolism to quinones. To protect against increased cytosolic DA where cytosolic antioxidants are no

longer sufficient, excess transmitter may be taken up into lysosomes and endosomes by VMAT action. Within these organelles, transferrin, lactotransferrin, and ferritin recycling could ensure sufficient iron to induce quinone formation.

NM is a dark brown insoluble compound with a structure composed of polymeric units with indole, catechol and benzothiazine rings (Crippa *et al.*, 1996; Zecca *et al.*, 1992; 1996). It appears in the DA neuron within three years of birth, reaches maximum levels in the seventh decade, and then slowly decreases (Foley and Baxter, 1958). It is probably degraded by H_2O_2 to form pyrrolecarboxylic and thiazolecarboxylic acids. NM is located in *melanosomes*, lysosomes with bilamellar membranes and dimensions of $\sim 1\,\mu m$ diameter (Duffy and Tennyson, 1965). The compound is particularly associated with the tertiary lysosomes known as lipofuscin granules, which appear to arise following lipid oxidation in the presence of amines (d'Ischia *et al.*, 1996). In particular, Barden showed that NM was colocalized with granules that stained for thiamine pyrophosphatase, which is classically associated with the *trans*-cisternae of the Golgi apparatus, and acid phosphatase, and is definitive for lysosomes (Barden, 1970). It should be noted that lysosomes and endosomes are related structures that both participate in secretory and degradative activity (Holtzman, 1992). As a lysosome constituent, NM could alter lysosomal function, such as lipid and protein breakdown and receptor-mediated endocytosis and associated recycling pathways.

The VMAT transporters responsible for synaptic vesicle DA accumulation are expressed in endosomes (Liu *et al.*, 1994). Since it seems likely that the ring charge of the quinones would preclude their effective recognition and/or sequestration as VMAT substrates, it seems that cytosolic DA is taken up into lysosomes and endosomes and that NM would be synthesized within these structures. This could protect against the accumulation of toxic levels of DA in the cytosol. In midbrain DA neurons, synaptic vesicles sequester ~ 3000–$14{,}000$ DA molecules, depending on conditions including the availability of L-DOPA (Pothos *et al.*, 1998). If a point is reached where vesicles are maximally filled, lysosomal VMAT would appear to be ideally situated to accommodate excess cytosolic transmitter. Of course, lysosomes also maintain an interior acidic pH gradient, which is required for VMAT action.

An estimate of the DA that could be sequestered by an endosome or lysosome expressing VMAT can be derived from a relationship identified for isolated chromaffin granules (Johnson, 1988)

$$\log[A]_{in}/[A]_{out} = \Delta\Psi F/RT + 2\Delta pH,$$

where A is the concentration of monoamine transmitter and $\Delta\Psi$ the transmembrane electrical gradient. If we assume that VMAT behaves in lysosomes as it does in chromaffin granules, a $\Delta pH = 5.2$ and $RT/F = +59\,mV$ predicts an equilibrium transvesicular DA gradient of at least $\sim 25{,}000:1$. Since melanosomes can be $1\,\mu m$ diameter, a single such organelle could easily sequester the free DA in the cytosol of a $10\,\mu m$ diameter cell body. Following conversion to NM, which would prevent the DA from leakage back across the lysosome membrane, the effective concentration gradient for DA and driving potential would remain high.

In addition to providing sequestration of excess DA, NM can play a protective role by generating stable complexes with metals like iron, copper, manganese, cadmium, mercury and others, and so block their toxic actions (Zecca *et al.*, 1994; 1996). Pesticides (Lindquist *et al.*, 1988) and the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (D'Amato *et al.*, 1986; 1987) are also adsorbed by NM. In summary, it may be that the NM pathway has evolved to help protect substantia nigra neurons from elevated cytosolic DA and DA–quinone throughout the decades of life. Perhaps this pathway could contribute to warding off SN degeneration and the development of PD.

Summary and Outlook

DA–quinone formation may provide the major step that mediates intraneuronal DA-dependent oxyradical stress. If the results with METH are generally applicable to DA neurodegeneration, actions of this product and its associated metabolites may explain the selective degeneration of DA neurons in PD and related conditions. The hypotheses we suggest in this article, that NM and LBs result from elevated cytosolic DA, one as a protective mechanism and the other as a result of interference with normal protein function, would help elucidate the neuronal responses to these chemical reactions and suggest preventative strategies for PD treatment. In particular, mechanisms for maintaining low cytosolic DA concentrations and increasing the ability of the cytosol to maintain the monoamine transmitter in a reduced form would be neuroprotective.

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