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Dopamine, Oxidative Stress and Protein–Quinone Modifications in Parkinson's and Other Neurodegenerative Diseases

Enrico Monzani, Stefania Nicolis, Simone Dell'Acqua, Andrea Capucciati, Chiara Bacchella, Fabio A. Zucca, Eugene V. Mosharov, David Sulzer, Luigi Zecca, and Luigi Casella**

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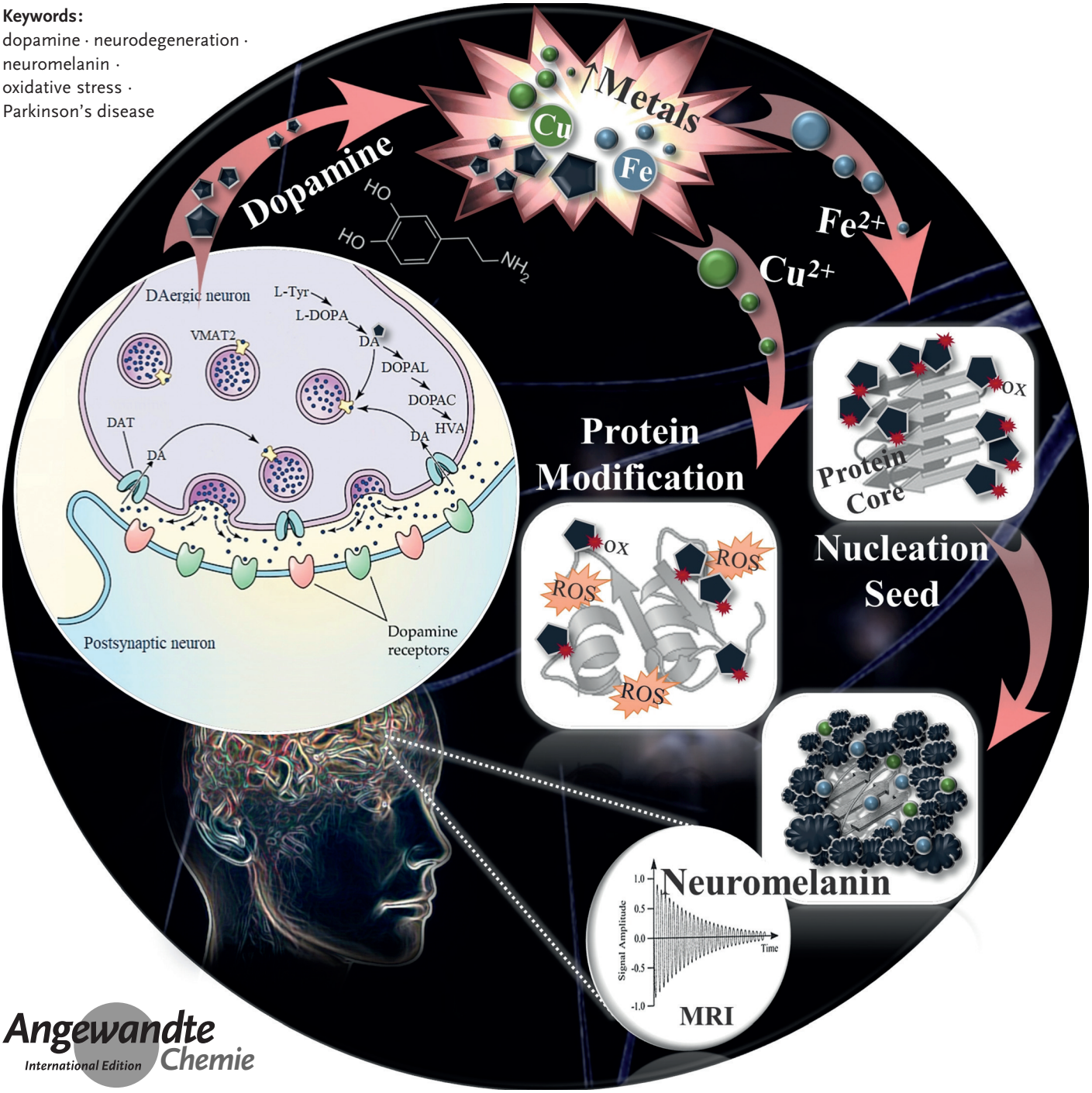

Dopamine (DA) is the most important catecholamine in the brain, as it is the most abundant and the precursor of other neurotransmitters. Degeneration of nigrostriatal neurons of substantia nigra pars compacta in Parkinson's disease represents the best-studied link between DA neurotransmission and neuropathology. Catecholamines are reactive molecules that are handled through complex control and transport systems. Under normal conditions, small amounts of cytosolic DA are converted to neuromelanin in a stepwise process involving melanization of peptides and proteins. However, excessive cytosolic or extraneuronal DA can give rise to nonselective protein modifications. These reactions involve DA oxidation to quinone species and depend on the presence of redox-active transition metal ions such as iron and copper. Other oxidized DA metabolites likely participate in post-translational protein modification. Thus, protein–quinone modification is a heterogeneous process involving multiple DA-derived residues that produce structural and conformational changes of proteins and can lead to aggregation and inactivation of the modified proteins.

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1. Introduction

The identification of dopamine (DA) as a neurotransmitter in the brain, made by Arvid Carlsson (Carlsson, Eric Kandel, and Paul Greengard were awarded the Nobel Prize in medicine and physiology in 2000 in part for their research on catecholamine neurotransmission),[1] was an important discovery as it had previously been considered to simply be a precursor in the synthesis of epinephrine (E) and norepinephrine (NE). DA depletion was firmly linked to Parkinson's disease (PD) by Hornykiewicz[2,3] in the 1960s, and it was subsequently established that DA-containing (DAergic) neurons were central to mechanisms underlying reward, cognition, and motor functions.[4,5] The three main DA projections—nigrostriatal, mesolimbic, and mesocortical—originate in the ventral midbrain and project to the striatum, the limbic system, and cortex, respectively. Loss of DA neurons in the *substantia nigra pars compacta* (SNpc), leading to a severe DA deficiency in the putamen and the caudate nucleus, produces the classical motor symptoms typical of PD: akinesia, rigidity, and tremor at rest.[6] Additionally, degeneration of locus coeruleus (LC) neurons, which utilize NE as neurotransmitter, occurs in PD and may precede that of SNpc DA neurons[7–10] and is thought to contribute to motor and non-motor symptoms of PD.[11] From the early studies of Hornykiewicz, it was soon recognized that PD patients could be treated with L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of DA, which is still the most effective drug for treatment of the disease and alleviates some of the motor symptoms.[12] L-DOPA treatment, however, does not halt the progression of PD, due to the ongoing loss of striatal terminals that store and release DA. It was argued whether L-DOPA treatment may be neurotoxic; however, it was demonstrated that motor fluctuations and dyskinesias in PD are not associated with the duration of L-DOPA therapy, but rather to disease progression itself. Therefore, there is no reason to delay the initiation of adequate L-DOPA therapy in patients with PD since L-DOPA treatment is safe and effectively relieves symptoms.[13–16] Other psychiatric and neurodegenerative disorders have been associated with altered DA metabolism, pointing to the necessity of maintaining DA homeostasis. Schizophrenia, a psychiatric disorder with a life-

[*] Prof. Dr. E. Monzani, Dr. S. Nicolis, Dr. S. Dell'Acqua, Dr. A. Capucciati, Dr. C. Bacchella, Prof. Dr. L. Casella
Department of Chemistry, University of Pavia
27100 Pavia (Italy)
E-mail: enrico.monzani@unipv.it
bioinorg@unipv.it

Dr. F. A. Zucca, Dr. L. Zecca
Institute of Biomedical Technologies
National Research Council of Italy
Segrate (Milano) (Italy)

Dr. E. V. Mosharov, Prof. Dr. D. Sulzer, Dr. L. Zecca
Department of Psychiatry, Columbia University Medical Center
New York State Psychiatric Institute
New York, NY (USA)

Prof. Dr. D. Sulzer
Departments of Neurology and Pharmacology
Columbia University Medical Center
New York, NY (USA)

The ORCID identification numbers for some of the authors of this article can be found under:
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time incidence of ≈1%, is characterized by an increase of amphetamine-evoked striatal DA release.[17,18] In addition, the DAergic system seems to be involved in the occurrence of cognitive decline, although the role of the dopamine system in Alzheimer's disease (AD) is still debated.[19] Another aspect of DA physiology emerging from recent studies is its role as a critical modulator of motivation, triggering immediate movements and learning, affecting future behavior.[20,21] Alterations in DA neurotransmission have been also identified in other neurodegenerative diseases besides PD, such as Huntington's disease and multiple sclerosis.[22] It should also be noted that DA is extensively produced in the body outside the brain, particularly in the human gastrointestinal tract.[23] We will only focus here on DA production and reactivity in the central nervous system (CNS).

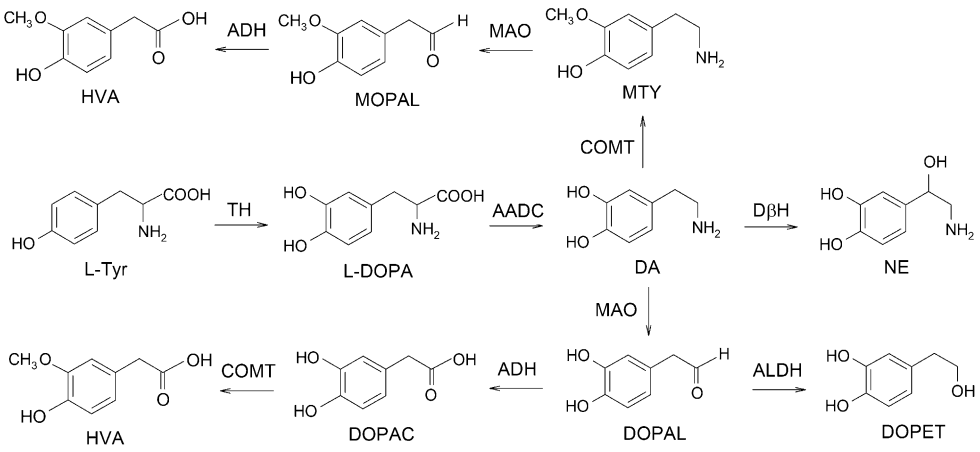
The specific chemical reactions of DA and related catecholamines (CAs), E and NE, make CA neurons (CAergic neurons) different from neurons producing other neurotransmitter types and particularly susceptible to oxidative damage. The loss of nigral DAergic neurons causes the characteristic tissue depigmentation recognizable in PD-diseased brains, due to the depletion of neuromelanin (NM), the dark substance accumulated in DAergic neurons by DA oxidative processes under physiological control.[24] Similar NM depletion occurs during PD, AD, and other neurodegenerative disease in LC neurons,[25,26] and probably in other brain areas, as NMs are distributed, albeit in smaller amounts, throughout the brain.[27] Importantly, NM formation represents, at least in the initial stage, a post-translational protein modification induced by CA-derived quinones as a protective process against quinone-related toxicity that arises as a consequence of oxidative stress. Such modifications occur, however, more generally and although they do not proceed to the extent seen in NMs, often produce more severe protein alterations than those caused by the common site-specific modifications corresponding to for example, phosphorylation,[28] acetylation,[29] and nitration[30] of protein residues. In addition, there is emerging evidence for the involvement of DA quinone (DAQ) modification in neuronal damage and in the progression of PD, and probably other neurodegenerative diseases.[18,22] Indeed, a continuous effort in neurodegeneration studies is in searching neurotoxic mechanisms common to different neurodegenerative diseases. Although PD and AD bear important differences, they also share a number of clinical, neuropathological features and neurochemical mechanisms. In addition to AD and PD there are several types of dementia and parkinsonism with overlapping characteristics thus showing also common pathogenic aspects, like the involvement of neuroinflammation, protein misfolding, metal toxicity, and CA modification of the involved proteins.

2. Dopamine Metabolism

The metabolism of DA is described in review articles;[31–34] here we will focus on DA metabolites that contribute to protein modifications. The main steps involved in DA biosynthesis and metabolism are outlined in Scheme 1. The principal route to DA in the CNS is from L-tyrosine, through hydroxylation of the phenol ring to L-DOPA by tyrosine hydroxylase (TH),[35] in an iron- and tetrahydrobiopterin (H_4-biopterin)-dependent process, followed by decarboxylation effected by aromatic acid decarboxylase (AADC).[36] Two other minor pathways to DA are known, one is cytochrome P450-dependent,[37] involving decarboxylation of Tyr to tyramine and then hydroxylation to DA, and the other involves oxidation of Phe to Tyr by phenylalanine hydroxylase, which belongs to the same enzyme family as TH and uses the same cofactors.[35] DA degradation occurs through oxidative deamination by FAD (flavin adenine dinucleotide)-dependent monoamine oxidase (MAO), a reaction producing 3,4-dihydroxyphenylacetaldehyde (DOPAL), hydrogen peroxide, and ammonia. Then, DOPAL is converted to the corresponding acid (DOPAC) by an aldehyde dehydrogenase (ADH)[38] and subsequently to homovanillic acid (HVA), a nonreactive metabolite, by catechol-*O*-methyltransferase (COMT).[39] COMT is also involved in a minor DA degradation pathway where DA is initially converted to 3-methoxytyramine (MTY) and subsequently by MAO to 3-methoxy-4-hydroxyphenyl acetaldehyde (MOPAL) and by ADH to HVA.[33] In turn, DA is the precursor of NE by the action of dopamine β-hydroxylase (DβH), a copper-containing monooxygenase.[40] The steps outlined in Scheme 1 do not occur in the same subcellular compartment and can actually occur in different cells, as shown schematically in Figure 1. In DAergic neurons,

Enrico Monzani obtained a degree in Industrial Chemistry at the University of Milano and a PhD in Chemical Sciences at the University of Pavia (Italy). Since 1997 he has held a permanent position and since 2010 he has been Associate Professor in Inorganic Chemistry at the University of Pavia. He is interested in bioinorganic chemistry, in particular in the activity of Fe- and Cu-containing enzymes and models and in the chemical processes during oxidative stress in biological systems.

Luigi Casella has been Full Professor of Chemistry at the Department of Chemistry, University of Pavia since 1990. He has served as the Chairman of the European COST Chemistry Action D21 "Metalloenzymes and chemical biomimetics", and the coordinator of other European projects in the field of bioinorganic chemistry. His main research interest has been the chemistry of metal enzymes involved in biological oxidations. More recently, his research has been directed toward understanding the molecular basis of diseases, which often involve the activation of oxidative processes causing damage to key cell components. He has also been the coordinator of national projects dealing with metal ion toxicity and neurodegeneration.



Scheme 1. Main pathways of DA biosynthesis and metabolism. Conversion of L-Tyr to DA is mediated by TH and AADC; DA is converted to DOPAL, DOPAC, and then HVA by MAO, ADH, and COMT, respectively. DA is, in turn, the precursor of NE by the action of DβH. DOPAL can be reduced to 3,4-dihydroxyphenylethanol (DOPET) by alcohol dehydrogenase (ALDH) according to a minor pathway. A secondary metabolic pathway of DA to HVA through MTY and MOPAL by COMT and ADH is also shown.

the vast majority of DA is stored in synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2); it has been suggested that the enzymes involved in DA biosynthesis, TH and AADC, are physically associated and interact with VMAT2.[41]

Upon excitation, the synaptic vesicles of DAergic neurons fuse with the plasma membrane and release DA to the extracellular milieu where it can interact with extrasynaptic DA receptors. Signaling is blocked by removing DA from the extracellular space. It can either be recycled after reuptake by DAergic neurons by the DA transporter (DAT), or degraded by MAO, which is present in both neurons and glial cells (Figure 1). It has been estimated that inside monoaminergic vesicles the concentration of DA and other CAs can reach values as high as 1 M,[15] and released DA can transiently produce low μM levels in the striatum (see Section 7).[42] The stability of DA within the vesicles is due to the acidic pH resulting from VMAT2 coupling to an vH^+-ATPase, which pumps protons into the vesicle.[43]

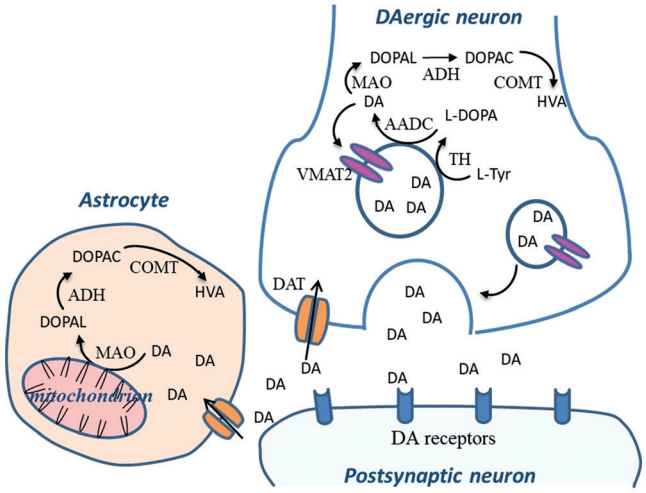


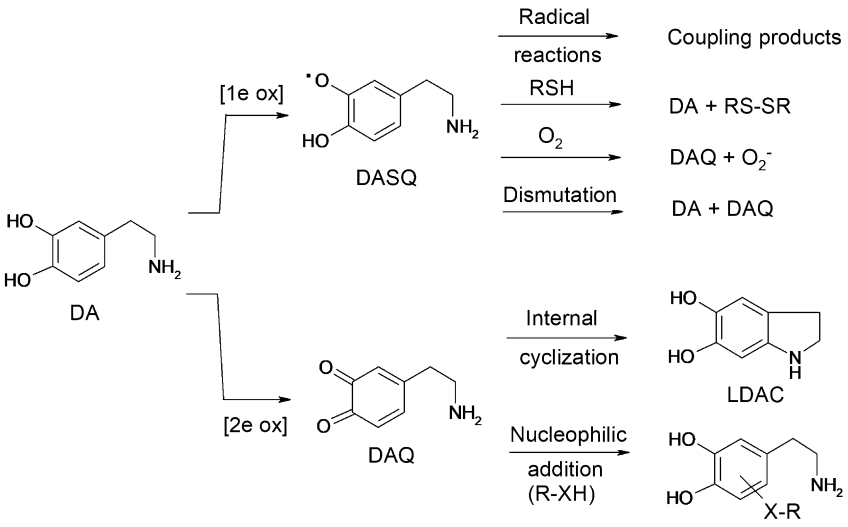
Figure 1. Main routes of DA metabolism in the brain. As DA is formed from L-DOPA, it is translocated into synaptic vesicles by VMAT2. MAO oxidatively deaminates the excess of DA in the cytosol, and the resulting DOPAL undergoes further catabolism, as described in Scheme 1. Upon neuronal excitation, DA is released into the synaptic cleft and interacts with postsynaptic DA receptors for signal transduction. Neuronal excitation stops upon re-uptake of DA through DAT into presynaptic neurons where it can be recycled for exocytosis, or imported and degraded by neighboring cells (both astrocytes, as shown in the figure, and microglia, since both possess degradative enzymes) to DOPAC and HVA.

3. Dopamine Reactivity and Its Metal-Catalyzed Oxidation

Midbrain DAergic neurons and their axon terminals are particularly sensitive to develop the pathological hallmarks of PD.[44] Dopamine can induce oxidative and nitrosative damage at axon terminals and their synaptic vesicles through a variety of mechanisms involving the production of reactive species. Redox active metal ions are certainly main promoters of these processes and among them copper and iron are by far the most important candidates. In this section, the main mechanisms of DA reactions under oxidative stress conditions of relevance in the context of PD will then be outlined.

3.1. Dopamine Reactions

The reactivity of DA largely depends on its relatively low free energy barrier to oxidation; indeed, DA undergoes a slow, spontaneous autoxidation, which is more significant as the pH is raised.[45] The presence of redox-active metals, such as Cu^{2+}[46] and Fe^{3+},[47] further promote DA oxidation, particularly when coordinated by suitable donor ligands, as discussed in Section 3.2. It is important to point out that in vitro studies on CA oxidation by metal ions are generally carried out at physiological pH, but several findings indicate that pH is more acidic (typically around 6.2–6.4) in brain areas affected by chronic diseases.[48,49] Copper and iron can promote CA oxidation due to their accumulation in both SN and LC tissues.[50,51] In PD a decrease of Cu and an increase of Fe levels is observed and this is the reason why it is commonly reported that the latter is mainly responsible for DA oxidation.[52] This can occur through one-electron and two-electron processes, which produce semiquinone radical (DASQ) or quinone (DAQ) species, respectively. The complex pattern of DA reactions is summarized in Scheme 2, which can be extended to the other CAs. Several intermediates produced by DA oxidation are reactive species that can

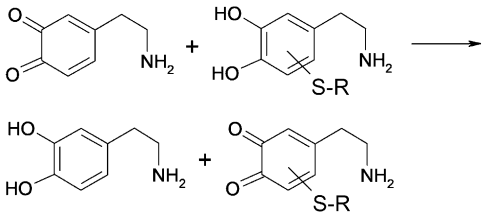


Scheme 2. Major reactions of the one-electron and two-electron oxidation products of DA to DASQ and DAQ.

modify proteins or oligomerize to melanic compounds. In vitro studies have shown that reaction pathways depend on reaction conditions and oxidizing agents, in particular the nucleophilicity of the groups involved and the reduction potential ($E°$) of the redox partners.[53] As the electron transfer is often followed by irreversible reactions, $E°$ values may not predict the preferred reaction path, whereas the pH of the medium and interaction with membrane play additional important roles. The redox potential of the DASQ/DA couple has not been determined, but should be close to that of the DAQ/DA couple, as the two redox steps of DA oxidation are not resolved in the cyclic voltammetry scans performed at different rates, which yield $E° = 0.75$ V for DAQ/DA ($DAQ + 2H^+ + 2e^- \rightleftarrows DA$).[54] At pH 7.4 the $E°'_{DAQ/DA}$ is ≈ 0.43 V but it increases slightly to ≈ 0.51 V at pH 5.5,[54] which can be considered as a lower limit in brain tissues with chronic diseases.[48,49]

Oxidation of DA to DASQ can be promoted in vivo by processes involving H_2O_2 activation by free heme or peroxidases[55] and prostaglandin H synthase.[56] The same oxidation can be mediated by activated microglia, since in this state microglia cells produce and release O_2^-, NO, and H_2O_2, together with pro-inflammatory factors.[57,58] DASQ is a reactive species and does not accumulate in solution. It can undergo coupling with other radicals, scavenging processes by reducing agents (i.e. glutathione (GSH) or other thiols), one-electron oxidation by O_2 to form DAQ and O_2^-, or disproportionation to DA and DAQ. The preferred pathway depends on the relative rate constants and concentration of the reagents. The rate dependence on [DASQ] is different for these processes. In particular, disproportionation is a second-order reaction requiring two DASQ radicals; it is expected to be rapid in vitro, but in vivo, where the concentration of DASQ is always low, it will have much less importance.

Dopamine may also be oxidized to DAQ in a two-electron process that does not generate DASQ. It is a much less common reaction since it requires an appropriate oxidant. Tyrosinase was earlier postulated to promote this reaction, as it is a key enzyme in melanogenesis.[59] However, tyrosinase is absent in DA neurons of human SNpc, and so its involvement in DA oxidation and NM biosynthesis in the brain is currently excluded.[60] In any case, DAQ, either formed from DA in a single step or through DASQ, is a reactive species toward reducing agents and nucleophiles (see Scheme 2). As the rate of nucleophilic addition to quinones depends both on the nucleophilicity of the nucleophile and the electrophilicity of the quinone, a correlation is found between the rate of nucleophile (GSH) addition to quinones and the $E°'$ of both semiquinone/catechol and quinone/catechol couples.[53] Thus, DAQ is more prone to undergo conjugate addition reactions than electron-richer quinones. DAQ is also a mild oxidant that could oxidize electron-rich catechols, such as those formed upon Michael addition of cysteine thiols to DAQ, in cross-oxidation type reactions (Scheme 3). The S-substituted quinone can undergo further Michael addition reactions leading to larger oligomers (see Section 6).



Scheme 3. DAQ cross-oxidation with electron-rich catechol adducts with nucleophiles, exemplified by a DA–thiolate adduct.

3.2. Metal-Catalyzed Dopamine Oxidation

As outlined in Section 3.1, redox metal ions catalyze DA oxidation in the presence of oxygen. In this context, the most relevant ions are iron and copper, due to their abundance in vivo and facility to undergo redox cycling. The presence of labile pools of these metal ions in the brain is probably a major source of this reactivity. Copper has an emerging role in cell signaling and neuronal excitability,[61] while the labile iron pool bears several regulatory functions,[62] and the susceptibility to iron dyshomeostasis is underscored by the observation that iron–β-amyloid (Aβ) complexes are abundant in plaque deposits in AD.[63] Additionally, labile iron accumulates in Lewy bodies of PD patients,[64] and more generally in brain areas affected by the disease.[65] The role of copper in PD pathogenesis is somewhat controversial. The levels of copper appear to be reduced in the SN and LC in PD cases.[52,66] On the other hand, the amount of copper found in the NM of SN and especially in that of LC is significant, suggesting that this metal actively participates in DA oxidative oligomerization.[51] In addition, recent studies show that

the production of reactive oxygen species (ROS) and neuronal toxicity induced by α-synuclein (αSyn) aggregates is entirely dependent on the presence of both copper and iron, and the effect is completely blocked by specific chelators of the two metal ions.[67] The connection with DA toxicity comes from studies showing that elevation of DA levels induces αSyn oligomers and increase nigrostriatal degeneration.[68] In the redox processes promoted by metal ions on DA, a variety of potentially toxic species such as DASQ, DAQ, O_2^-, H_2O_2, and hydroxyl radical can be formed through an intricate chain of reactions.[69]

The reactivity of metal ions towards catecholamines is modulated by binding to coordinating ligands and their association to peptides and proteins involved in neurodegeneration. In particular, besides αSyn, also Aβ, and the cellular prion protein (PrP) should be taken into account, as both Aβ and PrP display strong metal-binding properties.[70] In fact, it has been shown that DA promotes PrP aggregation, probably through the effect of DAQ/ROS species,[71] and it is likely that similar reactions will affect Aβ. In addition, both Aβ[72] and αSyn[73] interact with PrP, inducing cognitive impairment, and metal ions may well be involved in these interactions. According to systematic studies performed by our group, DA oxidation promoted by the copper complexes of these peptides share the same complex mechanism, described by the following sequence of reactions.[74–78] Assuming that these reactions will be particularly important intracellularly in the case of cytosolic DA excess and in oxidative stress conditions, or in the extracellular space when damaged neurons release large amounts of DA, the starting form of the metal ion will be oxidized, and this governs the interaction with the peptide. In the following scheme, we neglect competitive Cu^{2+} binding by DA ($K_d \approx 0.2$ μM),[79] which also gives rise to slow DA oxidation, to emphasize the promotion exerted by Cu^{2+} binding to peptides. Thus, binding of Cu^{2+} to the peptide [Reaction (1)], promotes DA oxidation [Reaction (2)], generating the Cu^+ species and DASQ.

$$Cu^{2+} + \text{peptide} \rightleftharpoons [Cu^{2+}\text{-peptide}] \quad (1)$$

$$[Cu^{2+}\text{-peptide}] + DA \rightarrow [Cu^{+}\text{-peptide}] + DASQ + H^+ \quad (2)$$

$$[Cu^{+}\text{-peptide}] + DA \rightleftharpoons [Cu^{+}\text{-peptide/DA}] \quad (3)$$

$$[Cu^{+}\text{-peptide/DA}] + O_2 \rightarrow [Cu\text{-peptide/DA/}O_2] \quad (4)$$

$$[Cu\text{-peptide/DA/}O_2] + H^+ \rightarrow\!\rightarrow [Cu^{2+}\text{-peptide}] + H_2O_2 + DASQ \quad (5)$$

$$2\,DASQ \rightarrow DA + DAQ \quad (6)$$

Reaction (4), between the Cu^+ complex and O_2 is the rate-limiting step of the process and requires previous coordination of DA to the complex [Reaction (3)] to occur. Reaction (5) is in competition with the release of O_2^- [Reaction (7)], depending on the affinity and binding rate of the Cu^+ complex to O_2, and superoxide is another source of H_2O_2, upon dismutation [Reaction (8)]. Hydrogen peroxide and Cu^+ can produce hydroxyl radical through the Fenton's Reaction (9).

$$[Cu\text{-peptide/DA/}O_2] + H^+ \rightarrow\!\rightarrow [Cu^{2+}\text{-peptide}] + O_2^- + DA \quad (7)$$

$$2\,O_2^- + 2\,H^+ \rightarrow O_2 + H_2O_2 \quad (8)$$

$$H_2O_2 + Cu^+ \rightarrow OH^- + OH^{\bullet} + Cu^{2+} \quad (9)$$

The overall process may thus generate a number of reactive species, including DASQ, DAQ, O_2^-, H_2O_2, and $OH^{\bullet}$, depending on the peptide involved. It should be noted that the binding mode of the peptide during catalysis might differ from the preferred coordination mode for Cu^{2+} and Cu^+ in resting conditions, due to redox cycling during the reaction. In addition, the reaction of H_2O_2 with Cu^{2+} species activates a further mechanism of DA oxidation.

The nature of the peptide is a major factor governing DA oxidation by Cu^{2+} ions according to the mechanisms outlined above. Both Aβ ($K_D \approx 0.58$ μM)[80] and the PrP peptide fragments containing the high-affinity Cu^{2+} binding site ($K_D \approx 10$ nM)[81] are strong promotors of Cu-mediated DA oxidation.[74–76] In the case of αSyn ($K_D \approx 0.20$ μM),[82] we found that it decreases Cu^{2+} reactivity,[77] but these experiments should be repeated using oligomers instead of monomeric αSyn, in view of the strong promotion of ROS production of αSyn aggregates in the presence of Cu^{2+}.[67] Redox cycling induced by DA on the copper–peptide complexes produces several modifications by DA-derived species of both Aβ and PrP peptides, as well as O-atom insertion at His and Met residues.[74,76,78,83] Interestingly, binding of the peptides to membranes has a quenching effect on Cu^{2+}-promoted DA oxidation, with the exception of Aβ, for which the inhibition is only partial.[78] The quenching effect of the membrane is due to the trapping of Cu^+ in the sites of the peptides containing a sequence with two close methionines, ^{1}MXXXM^5- at the N-terminal of αSyn, and -^{109}MXHM^{112}- in the fragment following the polar region comprising the octarepeats of PrP peptides. The rapid conformational rearrangement of the unstructured peptides occurring upon $Cu^{2+/+}$ redox cycling in solution is apparently prevented in the membrane, where the Cu^+-bound form is stable and reactivity is blocked. It should be noted that the peptides undergo competitive endogenous modification by the active species (DAQ and ROS) formed during the catalytic processes (see Section 5).

Much less is known about the effects of iron bound to neuronal peptides. Together with DA, iron is present in high concentration in DAergic neurons of the *substantia nigra* as NM–Fe complex.[51,84] The major iron-containing protein in the brain is ferritin (a multi-subunit iron-storage protein having H- and L-chains), which is particularly abundant in glial cells and at lower amounts in DAergic neurons.[51,85–88] Iron(III) forms complexes with the catechol moiety of DA which are more stable than those formed by copper(II), but are still sensitive to O_2, yielding DASQ, DAQ and other decomposition products.[89] A detailed study clarifies how the ratio between DA and Fe^{3+} or Fe^{2+} salts affects the redox reactivity, and that significant amounts of H_2O_2 (that likely arise by dismutation of superoxide) can be formed under appropriate conditions.[47] However, while several studies have addressed the binding of Fe^{3+} and Fe^{2+} ions to Aβ[90] and αSyn,[91,92] as well as the possible generation of ROS species,

data on DA oxidation promoted by Fe–peptide complexes are absent. In any case, iron plays an important role in NM formation through DA oxidation, as discussed in Section 6.

4. Dopamine Toxicity

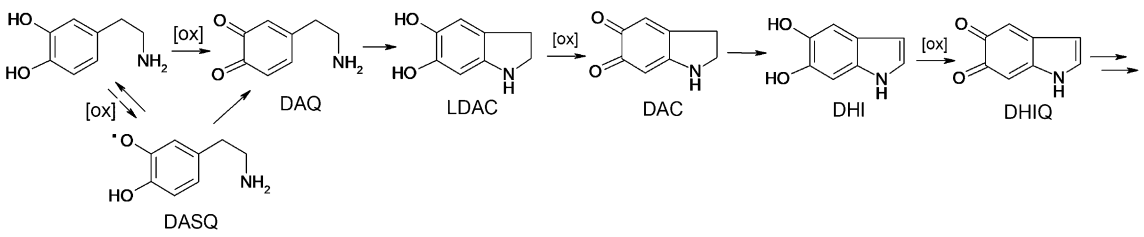
DA is widely recognized as a potential source of oxidative stress in the brain.[69] Excess cytosolic DA that is not cleared through catabolic enzymatic pathways (Scheme 1) or accumulated into synaptic vesicles can initiate reaction chains, induced by superoxide/DASQ and DAQ, both of which lead to neurotoxic effects. This scenario is typically encompassed by conditions of aberrant oxidative stress that characterize the initial events of the intricate cascade leading to neurodegeneration, including two important players that contribute to exacerbate DA toxicity: dyshomeostasis of redox-active metal ions[93] and NO. The toxicity of the latter is due to a variety of NO-derived species, including peroxynitrite and nitrite, the chemistry of which has been extensively reviewed.[30,94,95] Metal ion redox cycling and oxidative catalysis can be induced by the simultaneous presence of DA or its metabolites, H_2O_2 (produced both by DA oxidation by MAO and by superoxide dismutation), and endogenous reductants such as ascorbate.

Formation of DAQ triggers a reaction pathway associated with the process of melanogenesis,[96] although in the neuronal environment it is not driven to completion due to the variety of reactions competing with DA oxidative polymerization. The initial steps relevant to the present context of aberrant DA–protein modification are shown in Scheme 4.[97] The DAQ amino group yields an intramolecular cyclization to leukodopaminochrome (LDAC), an unstable intermediate easily converted to dopaminochrome (DAC).[43] The latter species is relatively stable and can accumulate before being further rearranged to 5,6-dihydroxyindole (DHI) and then oxidized to the corresponding quinone (DHIQ).[98] Therefore, a minimal list of DA-derived reactive species that can be considered responsible for post-transcriptional protein modifications includes DASQ, DAQ, DOPAL, DAC, and DHIQ. Of these, DASQ will essentially act as a precursor of DAQ, so that the main players are DAQ, DOPAL, DAC, and DHIQ. The reactivity of these species accounts for the effects commonly referred to as the toxicity of DA in the context of PD. In principle, the species responsible for protein modification can be recognized by accurate ESI/MS analysis of the protein fragment, at least when it is possible to trap the modified protein with just a single, or a few, DA-derived species. As discussed in Section 6, the modified catechol resulting from addition of a quinone to a nucleophilic residue is more electron rich than the starting quinone and subject to further easy modification. It has long been suspected that administration of L-DOPA during medical treatment of PD patients[12] could provide a relevant source of toxicity to be ascribed to catechol/quinone compounds.

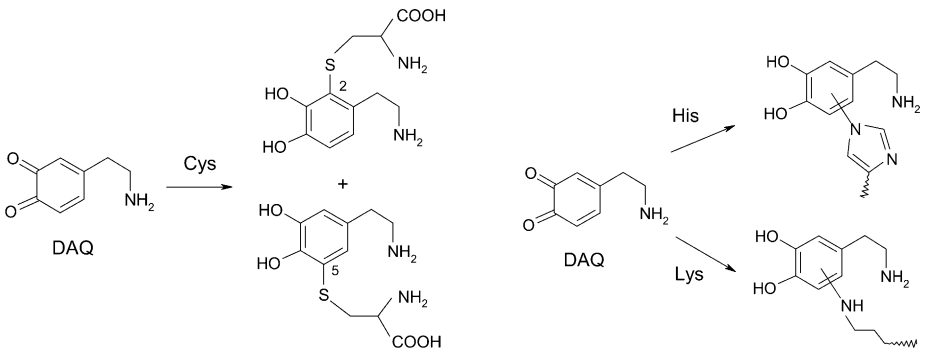
5. Dopamine Modification of Proteins and Peptides

Although the toxic effects of DA have long been recognized as due to protein modifications, little progress has been made in understanding the molecular basis and structural consequences resulting from these modifications. A limitation for the analysis of specific DA modification sites is the lack of suitable tools. Near-IR fluorescence has been recently introduced as a method for recognition of quinone-modified proteins,[99] but the technique does not clearly identify which sites are involved in the modifications. The same limitation applies to in vivo identification of proteins targeted by quinone reactive species through nitroblue tetrazolium (NBT)/glycinate redox-cycling staining after two-dimensional SDS-polyacrylamide gel electrophoresis.[100] Antibodies for DAQ-modified proteins would be useful but are not currently available. In addition, protein dopamination usually leads to insoluble aggregates that are difficult to analyze with HPLC/MS technique because they require preliminary proteolytic treatments with digestive enzymes, which may not reach their cleavage sites within the heterogeneous aggregates. For these reasons, although several reports deal with the effect of DA or L-DOPA on neuronal proteins as described below in this section, the type and sites of modification are often not unequivocally identified. The situation is more favorable when the effect of protein dopamination is studied in vitro at relatively short reaction times, when DA melanization of the protein sample is not extensive.

Recognition of the potentially toxic effect of the reactivity of DAQ species towards nucleophilic protein side chains dates to the mid-1980s with the work of Ito and Prota,[101,102] and later Dryhurst.[103] The polar groups of the side chains of Cys, His, and Lys can yield isomeric Michael addition products with DAQ that are generally difficult to recognize in protein adducts (Scheme 5). Studies in vitro performed with free amino acids and small peptides showed that in the case of Cys, the main DAQ adduct derived from nucleophilic attack of the thiol group at the C5 position of the quinone, but



Scheme 4. Initial steps of DA oxidation promoted by oxidizing species, generically indicated as [ox]. DAQ: dopamine quinone; DASQ: dopamine semiquinone; LDAC: leukodopaminochrome; DAC: dopaminochrome; DHI: 5,6-dihydroxyindole; DHIQ: 5,6-dihydroxyindolequinone.



Scheme 5. Michael type condensation products between DAQ species and nucleophilic amino acid side chains of Cys, His, and Lys. Isomeric products are shown for DA–Cys adducts only. In general, these can be characterized and separated from free amino acids or small peptides such as GSH, but it is difficult to differentiate between protein residues since HPLC/MS analysis of peptide fragments does not distinguish the isomeric derivatives.

also the C2 isomer, is usually obtained as a secondary product.[102,104] Conversely, the side chain of His preferentially attacks the C6 position of the quinone, but depending on reaction conditions in some cases also a small fraction of the C2 isomer has been observed.[105]

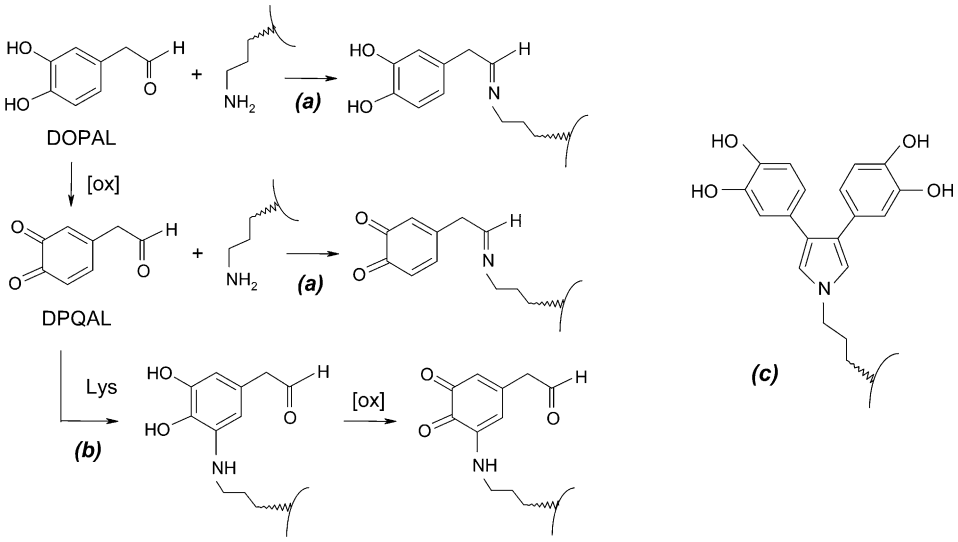
It is well known that the most reactive compounds in Michael additions to quinones are thiols in their ionized form. The corresponding reactions with amines, phenols, and catechols (including DA itself) are slower and depend on the pH of the solution since unprotonated amines, ionized phenols, and catechols, respectively, are effective nucleophiles. Therefore, the pK_a of the reacting group must be taken into consideration and this makes the His residues more reactive than Lys at pH 7.4. An important insight from these early studies is that peptide nucleophilic side chains bear a reactivity order of Cys $\gg$ His $>$ Lys towards DAQ species, and in particular the relative reactivity of the Cys thiol group with respect to the His imidazole group is of the order of 10^6.[106] Indeed, the reaction of DAQ with thiols is much faster than ring closure to LDAC.[107] It can therefore be anticipated that when a cysteine is accessible to DAQ species, it will be the primary site of quinone reaction and His residues will be affected only after reaction of the available cysteines. This has been demonstrated in a few instances when the sites of protein-bound DA could be identified.[108,109] Glutathione (GSH) is another obvious target of DAQ species, although the cytosolic content of GSH is much lower in neurons than in other cells.[69] It should be added that DA oxidation can trigger oxidative modification of amino acid residues, particularly methionines, as discussed below for αSyn.

A variety of neuronal proteins have been recognized to be inhibited or altered by interaction with DA-derived species and among those of relevance to PD αSyn is a high priority as it is the most abundant protein in Lewy bodies.[110] Indeed, early experiments showed that interaction of oxidized DA with αSyn leads to toxic oligomeric species,[111] although the nature of this interaction has not been clarified and may well be noncovalent.[112,113] The interplay between αSyn and DA-derived species is complex, because the protein regulates synaptic vesicle exocytosis[114,115] and has been claimed to regulate DA import into synaptic vesicles through an interaction with VMAT2[116] as well as DA reuptake by interaction with DAT,[117] and the regulation of αSyn function may depend on multiple reversible post-translational modifications.[115] A major reason that the DA interaction with αSyn is so elusive is that the protein contains no Cys, only one His (His 50), and 15 Lys residues in the sequence (140 amino acids). On the other hand, induction of toxic αSyn oligomers by interaction with DA has been unequivocally established by in vivo experiments.[68]

DA-modified αSyn may cause neurodegeneration by blocking chaperone-mediated autophagy degradation of αSyn and other cytosolic proteins.[118] Besides DAQ, other DA oxidized species that may be involved in the promotion of αSyn toxic oligomers are DAC,[119,120] DOPAC,[121] and DHIQ[122] (Scheme 4). In addition, Met oxidation of αSyn is systematically observed upon DA oxidation.[123–125]

The DA metabolite that has received most recent attention is DOPAL, as it forms covalent adducts with αSyn.[126–129] The Lys residues of this natively unfolded protein[130] are thus the primary target of DOPAL reactions, occurring at the aldehyde group and giving rise to reversible Schiff base condensation products, which in several cases could be characterized after reduction to stable amine derivatives (Scheme 6).[128,129] The majority of αSyn Lys residues were found to undergo DOPAL modification under various conditions, both in cell models and in vitro. In addition, DOPAL autoxidation results in the production of ROS and DOPAL–quinone (DPQAL), which can also react with Lys residues giving Michael addition products, as well as causing oxidation of Met residues. Surprisingly, DPQAL was not found to modify His 50. Another type of DOPAL modification was recently described from experiments with DOPAL and N-terminal acetylated αSyn, i.e., a dicatechol–pyrrole adduct with lysines (Scheme 6).[131] This modification corresponds to the addition of two DOPAL molecules to the Lys side chain through their aldehyde groups and the formation of a new carbon–carbon bond between their alkyl chains to form the pyrrole ring. It is important to emphasize that both DA- and DOPAL-modified αSyn inhibit the formation of mature amyloid αSyn fibrils, and the resulting oligomers have different sizes and different morphologies.[132] Among the toxicity mechanisms ascribed to αSyn aggregates, mitochondrial dysfunction,[133] autophagy impairment,[134] and membrane damage[135] have been proposed, but it is still unclear which are the most relevant aggregates for the pathology. The special reactivity and high toxicity of DOPAL prompted the so-called "catecholaldehyde hypothesis" for the pathogenesis of PD.[34]

Several other proteins have been reported to be modified by DA, although the type and sites of modification are often incompletely characterized. Among these proteins, glucocer-



Scheme 6. DOPAL-promoted modifications of αSyn. Pathways (a) involve Schiff base formation between DOPAL, or DPQAL, and Lys residues, while pathway (b) corresponds to Michael addition between DPQAL and Lys. Compound (c) is the covalent dicatechol pyrrole adduct between two molecules of DOPAL and a Lys residue.

ebrosidase (GCase), parkin, and DJ-1 are of high priority in view of their association with oxidative stress in PD.[136] GCase (also known as acid-β-glucosidase) is a lysosomal enzyme catalyzing the cleavage of the glycolipid glucosylceramide to ceramide and glucose;[137] this enzyme is deficient in a lysosomal storage disorder, Gaucher disease, but when single-mutant alleles are present they have a strong association with PD, and are the most widespread genetic risk factor for parkinsonism identified to date.[138,139] Recently, it was shown[140] that DA oxidation to DAQ produced covalent modification of GCase at the active site Cys residues,[141] resulting in reduced enzyme activity, lysosomal dysfunction, and αSyn accumulation. Intriguingly, αSyn and GCase form a protein complex in solution at lysosomal pH 5.5, with K_d in the μM range, which dissociates at pH 7.4.[142] Inactivation of parkin by covalent DA modification at active Cys site has been reported more than a decade ago.[143] Parkin is an E3 ubiquitin ligase, involved in the ubiquitin post-translational modification of substrate lysines in proteasomal and autophagic protein degradation pathways.[144] Understanding its function is crucial for treatment of early onset autosomal recessive PD because the majority of cases are linked to mutations in the parkin gene (*PRKN*).[145] DJ-1 plays an incompletely defined neuronal protective role against oxidative stress,[146] and apparently uses regulatory Cys oxidation in peroxiredoxin-like scavenging of H_2O_2, and highly oxidized DJ-1 was found in the brains of PD patients.[147] Together with other proteins, DJ-1 was found to be DAQ modified in proteomic experiments of mitochondria exposed to ^{14}C-DA,[148] and the modifications were identified to occur at Cys 106 and Cys 53.[149] Modification of DAT occurs at some of the 13 Cys residues of the human protein and results in an inhibition of DA uptake by the protein.[150] A recent X-ray structural analysis of the DAT–DA complex, with protein from *Drosophila melanogaster*, does not show the presence of Cys residues in the DA binding site,[151] suggesting that while the DAQ modification may be peripheral, it induces severe structural changes in the protein. Other proteins that have been reported to undergo interactions and possibly modification by DA derivatives can be found in the review papers by Segura-Aguilar and co-workers,[43,120] but in most cases the DA derivative responsible and the protein sites undergoing modification have not been identified.

6. Dopamine in Neuromelanin Biosynthesis

The formation of NM pigments provides a protective mechanism that prevents neurotoxicity induced by the accumulation of cytosolic CAs that are not sequestered into monoaminergic synaptic vesicles by VMAT2.[41,152] The DAergic regions with higher VMAT2 expression (SNpc < *substantia nigra pars reticularis* < ventral tegmental area (VTA)) show a corresponding lower rate of NM synthesis and decreased vulnerability of DA neurons in PD (SNpc > *substantia nigra pars reticularis* > VTA). Then, vulnerability of DA neurons in PD is related to their NM content.[153,154] The biosynthesis of NMs is initiated by catecholamine oxidation to reactive quinones, either by autoxidation or far more likely, metal-catalyzed oxidation,[155] and is favored by the presence of seeds of aggregated amyloid proteins and peptides with which the quinones rapidly react.[24] There is no evidence for the involvement of enzymatic reactions in the DA oxidation process to NM, and tyrosinase, responsible for the formation of peripheral melanins,[156] was not found in a proteomic analysis of NM.[60] The protective effects of NMs can thus be ascribed to the elimination of reactive quinones from the cytosol, which are then bound as units of the growing melanic component of the melanin–protein conjugate and trap potentially toxic metal ions, particularly iron and copper, by chelation in the NM matrix. The initial melanin–protein conjugate is further elaborated by attachment of lipid components and sequestered within specialized cytoplasmic NM autophagic-lysosomal organelles surrounded by a double membrane.[24,27,60,157]

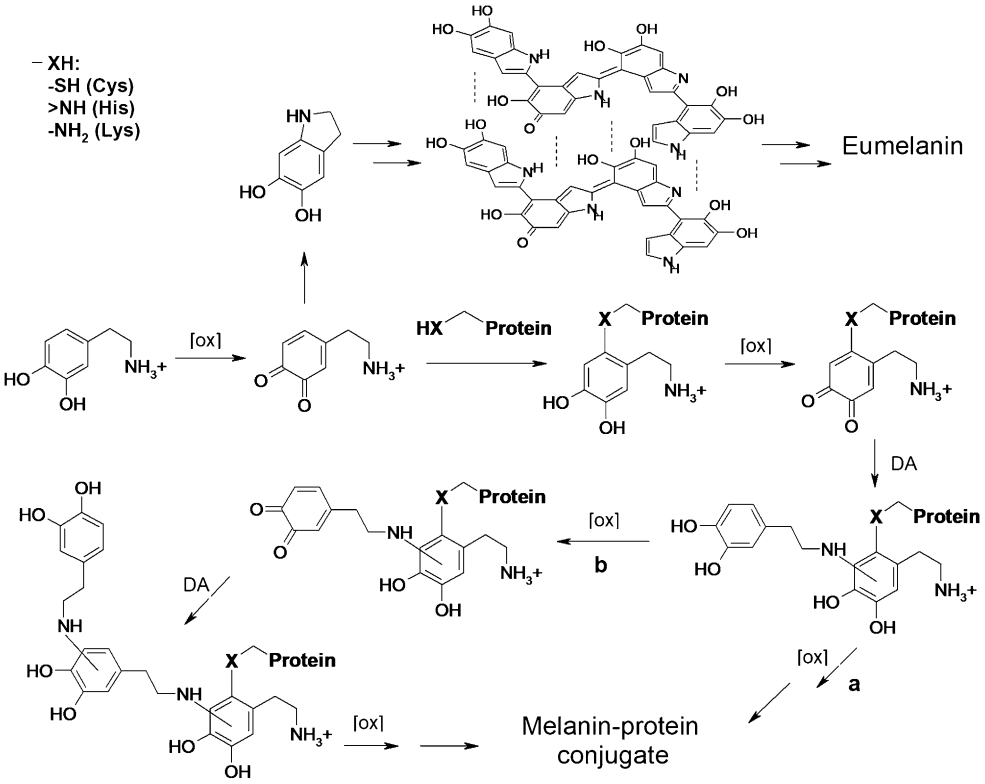
The initiating event of the NM biosynthetic pathway can be traced to the presence of cytosolic seeds of oligomeric proteins or aggregated peptides organized in amyloid cross-β structures and containing exposed Cys (and likely His) side chains, which act as traps for quinone species. Synthetic NM models developed using fibrillar and native lactoglobulin indeed show that the compact fibrillar structure of the protein makes the side-chain thiols on the fibril surface more easily accessible to DAQ than in the native protein.[158] The presence of an amyloid protein/peptide core in NM is confirmed by X-ray diffraction studies of the NM pigments extracted from several brain areas; these studies invariably show the characteristic pattern of 4.7 Å typical of cross-β structure,

related to the distance between β chains in the protein backbone.[27] This is the only structural element detectable in human NMs, while completely absent is the signature of π-stacked layers of polydopamine oligomers (with 3.5 Å separation)[159] present in DHI melanin and in peripheral melanins.[160] The lack of structured melanin in NM depends on the mechanism of formation of the polymer, which involves the progressive addition of DA residues to electron rich DAQ–protein addition products (Scheme 7). Protein modifications with DHI or DHIQ units have not been found so far in the melanin–protein conjugates, perhaps because the initial reaction of DAQ with exposed nucleophilic residues is faster than cyclization of DAQ to LDAC (Scheme 4).

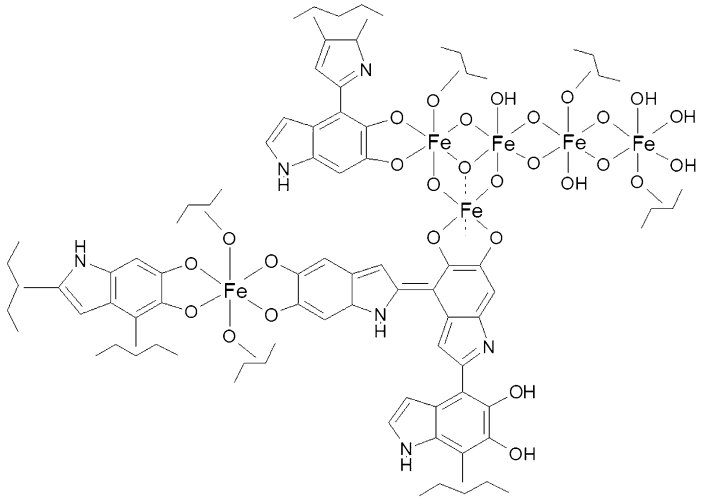
A clue to understanding how the unstructured melanic moiety of melanin–protein conjugates progressively grows in during the reaction with DA/DAQ comes from our study of DA modification of myoglobin[104] and was subsequently confirmed through the preparation of similar conjugates between DA and serum albumin[109] and lactoglobulin.[158] The process starts with the addition of a nucleophilic protein residue to DAQ and proceeds with consecutive additions of DA molecules to the growing chain of the melanin–protein conjugate. Note that the DA addition products, Cys-DA, His-DA, and Lys-DA (Scheme 5), are more easily oxidized to quinones (Protein-X-DAQ) than DA to DAQ. Therefore, in the subsequent step another DA molecule adds to the Protein–X-DAQ intermediate, allowing the stepwise growth of the DA melanin–protein conjugate (Scheme 7). This is confirmed by the CID/MS analysis of melanin–myoglobin precipitate, where the peptide 80–96 was found modified by 5 DA units at His81/His82 cluster and 4 DA units at His93 (heme proximal ligand; numbering refers to the horse heart sequence). When the protein contains accessible Cys, as in serum albumin,[109] neuroglobin,[161] and lactoglobulin,[158] the DA oligomerization occurs primarily at these residues. In Scheme 7, the competitive pathway leading to DA melanin (eumelanin) is also indicated. This would lead to the stacked layers of DA oligomers, the presence of which has been observed occasionally as a minor component in synthetic NM models, through the characteristic X-ray powder signature.[24,27,159]

Chemical degradation studies of NM pigment of SN with H_2O_2, HI, and HCl yielded products typical of Cys–DA–melanin, DA–melanin, and protein-bound DA–melanin degradation, which suggest the presence of a significant portion of eumelanic component in NM, together with a lower fraction of pheomelanin (approximately a 3:1 eumelanin/pheomelanin ratio).[27,162] If this is the case, we assume that a yet unknown, unstructured type of eumelanin must be present in human NM. Chemical degradation studies also reveal signatures of contributions by different DA metabolites to the melanic fraction of NMs.[163]

Iron has a role in promoting cytosolic DA oxidation and remains trapped in the melanin component by chelation to the network of catecholic groups present in the melanic polymer. Indeed, NM isolated from SN contains significant amounts of iron,[27,51,164] which is present both in aggregated form (EPR-silent) as iron(III) oxo/hydroxo clusters similar to those present in ferritin,[165] and in mononuclear paramagnetic (EPR-detectable) sites (Scheme 8). In the NM from LC, in addition to iron, copper is present in appreciable amounts,[51] indicating that copper is also a promotor of CA oxidation, which in this case would consist of both NE and DA with their metabolites.[166] The NM-bound Cu^{2+} exhibits a weak EPR signal and, similarly to Fe^{3+}, may be trapped in the melanic fraction.[51] In NMs isolated from different brain regions there are differences in the melanin components as well as in the quantitative melanin/protein ratios,[167] which reflect the specificity of neuron types and CA precursors (mainly DA in SN, DA and NE in LC, and other CAs in putamen,



Scheme 7. Initial steps of the NM formation process by covalent conjugation of DAQ/DA to protein as revealed in studies on synthetic melanin–protein conjugates (bottom pathway). The growing chain of the melanic portion is unstructured. The competitive eumelanin formation by DA oxidative polymerization leading to stacked melanic layers is also shown (top pathway).



Scheme 8. Schematic representation of the iron centers present in NM of SN. Iron is bound to the melanic component of NM and distributed into two site types: a) mononuclear, EPR-detectable sites of high spin, six-coordinated iron(III) centers of rhombic symmetry and b) multinuclear clusters of strongly coupled (EPR-silent) iron(III) centers linked by oxo-hydroxo bridges, which bear resemblance with the more extended aggregate present in the ferritin iron core. The multinuclear clusters are strongly bound and buried within the pigment, whereas mononuclear iron centers are bound to more exposed, low-affinity sites, which become more appreciably populated in conditions of iron overload.[24]

cortex, cerebellum, and other regions).[27,163,166,167] In any case, the complex substance resulting from covalent assembly of melanin, metal ions, and protein moieties that is not degraded by proteasome in the cytosol is engulfed into autophagic vacuoles which fuse with lysosomes. Lysosomal proteases are unable to degrade NM precursors, which are further modified due to continuing fusion of the autolysosome with other autophagic vacuoles and lysosomes that contain different lipids and proteins. The final NM-containing organelle surrounded by a double membrane is a special autolysosome 0.5–3.0 μm in size that contains a protein matrix, NM pigment, and lipid bodies (Figure 2).[24,27,60,155,157]

Interestingly, NM and Aβ share important characteristics as they are both insoluble and accumulate with aging in the brain with aging. Metals are involved in their synthesis and both NM and Aβ bind metals, thus playing a protective role against metal toxicity. Their synthesis removes toxic compounds like oligopeptides and excess cytosolic DA. In extracellular milieu NM and Aβ can be neurotoxic by activating microglia with consequent neuroinflammatory and neurodegenerative effect.[168]

7. The Relationship between Neuromelanin and Cytosolic Dopamine

The concentration of cytosolic CAs and their oxidation regulates NM synthesis, and early studies suggested that accumulation of cytosolic DA may lead to neurodegeneration.[169] The only means to measure cytosolic CA levels directly is by "intracellular patch electrochemistry" (IPE), a technique that uses a patch pipette to break into a cell and, within that pipette, a carbon fiber electrode that oxidizes the CA. If run in a cyclic voltammetry mode, this technique can distinguish several catechols.[170–172] L-DOPA vastly increases cytosolic CAs, in PC12 cells to ≈25 μM,[171] and in mouse and human neurons to 10–20 μM. Interestingly, L-DOPA exposure produces much higher levels of cytosolic DA in neurons from SNpc than VTA, another brain DAergic population that is spared in PD. This difference was attributed to Ca^{2+}-dependent activation of DA synthesis via AADC and the fact that cytosolic Ca^{2+} reaches higher levels in SN neurons

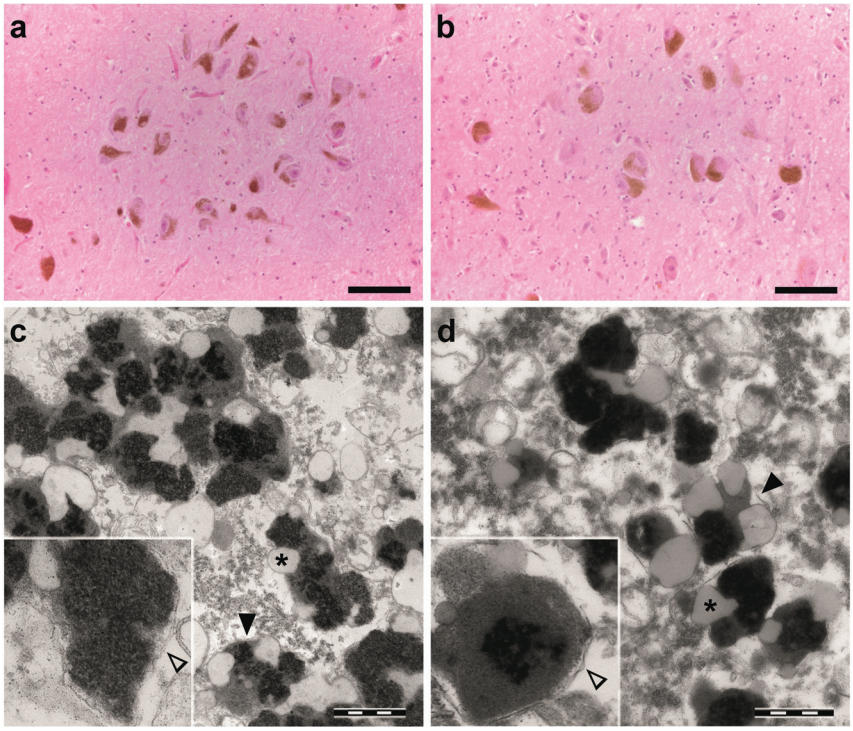


Figure 2. NM-containing organelles of human SN and LC. a) SN and b) LC tissues of a healthy 71-year-old subject. Hematoxylin and eosin staining shows many neurons whose cytoplasm is highly enriched with the NM pigment (black-brown colored). Scale bar in both panels = 100 μm. c,d) Transmission electron microscopy shows the classical structure of NM-containing organelles present both in c) SN and d) LC neurons. These special organelles are membrane-bounded (black arrowhead in (c) and (d)), contain large amounts of NM pigment (black and electron-dense) which is closely associated with lipid bodies (asterisk) and a protein matrix. In the higher magnification insets, a double membrane delimiting the NM-containing organelles (empty arrowhead in each inset) is clearly visible in both brain areas. c) SN of a healthy 89-year-old subject; scale bar = 1 μm. d) LC of a healthy 81-year-old subject; scale bar = 1 μm. For tissue treatments and ethics policies refer to Refs. [27, 60, 167].

due to the activity of the $Ca_v1.3$ L-type calcium channels that are involved in generating pacemaking activity in SN but not in VTA neurons.[172–174] Consistent with this difference in DA homeostasis, human SNpc neurons produce higher NM levels than VTA DA neurons.[153] Exposure of rat-cultured SNpc DA neurons to high μM concentrations of L-DOPA for several days is sufficient to produce NM that can be detected as a visual pigment, under electron microscopy or by EPR.[152] As cytosolic DA is neurotoxic, exposure to L-DOPA also caused dose-dependent neuronal death.[172] Overexpression of the PD-associated protein αSyn can also increase cytosolic DA, apparently by disrupting membranes and making storage vesicles leaky,[111,171,175,176] although it is unknown if this occurs under disease-related conditions.

Measurements of DA stores indicate that cytosolic DA levels must be maintained at low concentrations for neuronal health. NM synthesis occurs when cytosolic DA levels are greater than 3 μM, but when cytosolic DA exposure (i.e. concentration × time) becomes too high the neurons die, although chromaffin cells are far less susceptible to cytosolic CA toxicity. There are multiple means that the cells use to maintain low levels of cytosolic CA, including VMAT2-mediated sequestration in synaptic vesicles, catabolic breakdown by MAO (although MAO inhibition can be protective in these systems, possibly by blocking the synthesis of DOPAL),[172] allosteric feedback inhibition of TH by DA, and quite likely by antioxidant responses including GSH.

Synthesis of NM appears to provide another means to protect neurons from the consequences of excess DA in the cytosol, although this, to our knowledge, has not been formally demonstrated by blocking the formation of NM-containing organelles. It is not known whether the levels of L-DOPA administered to PD patients exacerbate NM synthesis; however, if it does, this may not reflect toxic consequences of the drug, as NM synthesis may be sufficient to protect against deleterious responses.

8. Magnetic Resonance Imaging of Neuromelanin as a New Tool for Diagnosing Parkinson's and Alzheimer's Diseases

Magnetic resonance imaging (MRI) of NM in SN has become an important method for diagnosing PD as shown by many studies in the last 12 years (for a review see Ref. [177]). Indeed, in PD there is selective loss of DA neurons containing NM and a decrease of NM pigment concentration in SN of PD patients.[178] There is also a growing interest in MRI of NM in LC for the early diagnosis of AD and PD.[179] In fact, LC neurons loss is the first event occurring in AD.[180] MRI studies of NM in LC were limited in the past by the small size of this region that could be poorly imaged with 3T MRI scanners. Nowadays, the availability of 7T scanners has increased and a number of MRI studies of NM in LC have been reported.[181]

In DA neurons of SN, and NE neurons of LC, the NM pigment is always present as a complex with Fe and/or Cu.[51] As shown in Section 6, the Fe sites are heterogeneous in NM of SN and only a minor fraction of mononuclear iron(III) is EPR detectable at low temperature, although also the EPR-silent Fe clusters may access excited paramagnetic states at physiological temperature and hence contribute to MRI signal amplification. Regarding the Cu sites of NM of LC, a similar situation may be present. In any case, for the NM of SN it was demonstrated that relaxation rates in MRI for the complex formed by melanic component of NM with Fe are linearly related to their concentration.[182] Moreover, in slices of human midbrain it was observed that the MRI signal of NM is linearly related to the concentration of NM in SN, thus providing a qualitative and quantitative validation of MRI of NM.[183] This shows that MRI of NM is a reliable marker of NM concentration, which in turn is a marker of CA neuron number. Based on the accurate knowledge of NM components in terms of melanic, lipid, and protein moieties, and the metal composition, future studies will be conducted for the development of specific MRI sequences for NM detection. Considering that DA– and NE–protein adducts are structural components of NM,[27,166] these are being used for elaborating dedicated sequences for high-resolution imaging of NM-containing neurons in SN and LC. These new sequences will hopefully allow to image the early loss of NM neurons in PD and AD patients, that is, to diagnose the disease years before the onset of symptoms, so that neurorescuing therapies can be applied in due time.

9. Summary and Outlook

Protein dopamination is an important and yet comparatively unrecognized post-translational modification of proteins and peptides in the brain. This is a functional outcome of high levels of cytosolic DA and has long been hypothesized to play an important role in PD pathogenesis.[171,172] It is usually dependent on DA oxidation products, mostly DAQ, and its reactive metabolites, such as DOPAL and DPQAL, and therefore its toxic effects are linked to conditions of oxidative stress. Protein–DA modifications can occur intra- and extracellularly, following DA exocytosis. Under normal conditions, the effects are limited to small amounts of DA leakage from DA vesicles, and it is handled by binding to Cys residues of GSH or nucleophilic residues of other peptides, particularly when these are part of oligomers of β-sheet structure, where the side chains are exposed to the surface and more easily accessible to external reagents. The DA covalent modification is then used for building the precursors of NM particles, which will result upon further elaboration through the autophagic–lysosomal pathway; this represents a neuroprotective mechanism for clearance of potentially reactive molecules.[24,27,60,155] While the pathway leading to NM is better clarified because NM can be isolated and characterized, the effects of DA modification of other proteins or peptides remain poorly understood. We anticipate that DA reaction with proteins containing suitable nucleophilic residues is much more general and becomes dramatic in conditions of chronic inflammation and release of large amounts of DA from damaged neurons, as it occurs in PD. To date the interaction between DA-derived species has been studied for only a few proteins, although a number of proteins likely form such conjugates. These conjugates likely play a functional role

not yet characterized, thus deserving investigation. Another important aspect, related to NM biosynthesis and structure, is the need for better characterization of the melanic components of the protein assemblies of NM and their metal-binding properties. This knowledge is key to gain an understanding of the MRI effect and the individual dependence of the signal amplification. Further investigation of the structure of melanic component in NM would provide useful information to generate MRI sequences specific for NM that will be employed to produce higher resolution images of neurons in SN and LC relevant for the early diagnosis of PD, AD, and related neurodegenerative diseases.

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Conflict of interest

The authors declare no conflict of interest.

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