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REVIEW

Protective and toxic roles of dopamine in Parkinson's disease

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Abstract

The molecular mechanisms causing the loss of dopaminergic neurons containing neuromelanin in the substantia nigra and responsible for motor symptoms of Parkinson's disease are still unknown. The discovery of genes associated with Parkinson's disease (such as alpha synuclein (SNCA), E3 ubiquitin protein ligase (parkin), DJ-1 (PARK7), ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL-1), serine/threonine-protein kinase (PINK-1), leucine-rich repeat kinase 2 (LRRK2), cation-transporting ATPase 13A1 (ATP13A), etc.) contributed enormously to basic research towards understanding the role of these proteins in the sporadic form of the disease. However, it is generally accepted by the scientific community that mitochondria dysfunction, alpha synuclein aggregation, dysfunction of protein degradation, oxidative stress and neuroinflammation are involved in neurodegeneration. Dopamine oxidation seems to be a complex pathway in which dopamine *o*-quinone, aminochrome and 5,6-indolequinone are formed. However, both dopamine *o*-quinone and 5,6-indolequinone are so unstable that is difficult to study and separate their roles in the degenerative process occurring in Parkinson's disease. Dopamine oxidation to dopamine *o*-quinone, aminochrome and 5,6-indolequinone seems to play an important role in the neurodegenerative processes of Parkinson's disease as aminochrome induces: (i) mitochondria dysfunction, (ii) formation and stabilization of neurotoxic protofibrils of alpha synuclein, (iii) protein degradation dysfunction of both proteasomal and lysosomal systems and (iv) oxidative stress. The neurotoxic effects of aminochrome in dopaminergic neurons can be inhibited by: (i) preventing dopamine oxidation of the transporter that takes up dopamine into monoaminergic vesicles with low pH and dopamine oxidative deamination catalyzed by monoamino oxidase (ii) dopamine *o*-quinone, aminochrome and 5,6-indolequinone polymerization to neuromelanin and (iii) two-electron reduction of aminochrome catalyzed by DT-diaphorase. Furthermore, dopamine conversion to NM seems to have a dual role, protective and toxic, depending mostly on the cellular context.

Keywords: 5,6-indolequinone, aminochrome, dopamine, dopamine *o*-quinone, neurodegeneration, neuromelanin, Parkinson's disease.

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Dopamine metabolism

Dopamine synthesis and storage

Dopaminergic neurons are involved in motor activity, in which dopamine is synthesized, stored and released to intersynaptic space. Dopamine is synthesized from the amino acid tyrosine in two steps that occur in the cytosol: (i) hydroxylation of tyrosine to l-dihydroxyphenylanaline (l-dopa), a reaction catalyzed by tyrosine hydroxylase (TH) that requires oxygen and (ii) decarboxylation of l-dopa to dopamine, a reaction catalyzed by aromatic amino acid decarboxylase (AADC) that generates CO_2. Interestingly, dopamine synthesis does not result in dopamine accumulation in the cytosol as a consequence that TH and AADC are associated with the vesicular monoaminergic transporter-2 (VMAT-2) generating a complex where tyrosine is converted to l-dopa that immediately decarboxylated to dopamine. The

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Abbreviations used: AADC, aromatic amino acid decarboxylase; COMT, catechol *ortho*-methyltransferase; GSTM2, glutathione *S*-transferase M2-2; l-dopa, l-dihydroxyphenylanaline; MAO, monoamine oxidases; NM, neuromelanin; TH, tyrosine hydroxylase; VMAT-2, vesicular monoaminergic transporter-2.

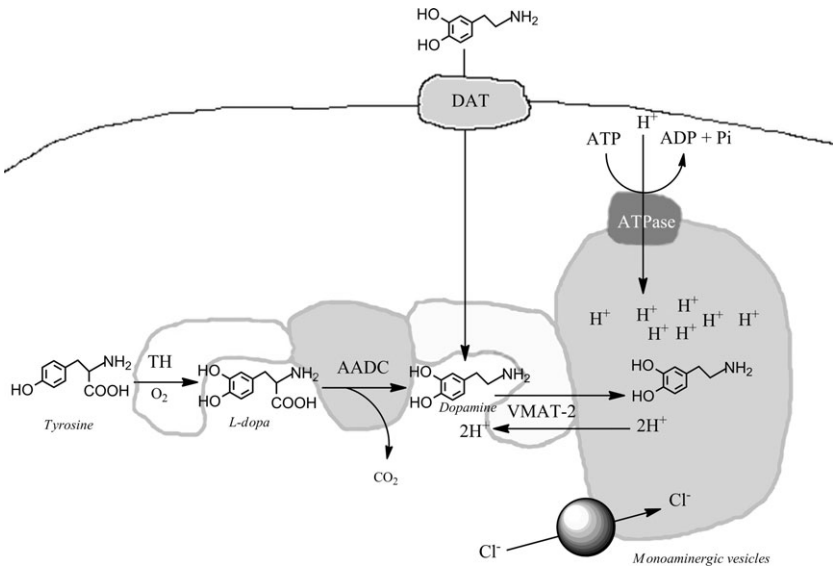

Fig. 1 Dopamine synthesis. In the presence of oxygen tyrosine hydroxylase (TH) catalyzes the conversion of the amino acid tyrosine to l-dopa that it is substrate for aromatic amino acid decarboxylase (AADC) that catalyzes the formation of dopamine and CO_2. TH and AADC enzymes form a kind of complex with the vesicular monoaminergic transporter-2 (VMAT-2) that is localized in the membrane of monoaminergic vesicles. The complex of TH-ADDC-VMAT-2 prevents the release of dopamine into the cytosol since the formed dopamine is directly transported into monoaminergic vesicles. The monoaminergic vesicles have an ATPase that pump up protons into the vesicles using ATP, generating a pH decrease and proton gradient that is coupled to dopamine uptake mediated by VMAT-2 because for one dopamine molecule uptake into the vesicles one proton is released to the cytosol. The low pH inside the monoaminergic vesicles is essential to store high concentration of protonated dopamine hydroxyl groups.

latter is taken up into the monoaminergic synaptic vesicles by VMAT-2 (Cartier *et al.* 2010), a dopamine transporter localized in the membranes of these vesicles (Miller *et al.* 1999). This complex (TH-AADC-VMAT-2) seems to play an important role in the prevention of dopamine oxidation as the protons of the hydroxyl groups are dissociated when dopamine is found in the cytosol at pH 7.4 (Linert *et al.* 1996). Therefore, dopamine uptake into monoaminergic vesicles prevents both the accumulation of free dopamine in the cytosol and the oxidation of dopamine to *o*-quinone because the pH inside monoaminergic synaptic vesicles is 2.0–2.4 pH units lower than the pH in the cytosol (Guillot and Miller, 2009). At this low pH, the protons of dopamine hydroxyl groups are strongly bound to the oxygen of hydroxyl groups. The low pH of monoaminergic vesicles is because of a VMAT-2-coupled vesicular ATPase, which hydrolyzes ATP to ADP, inorganic phosphate and one proton (H^+), creating a proton gradient (Fig. 1).

The dopamine stored in monoaminergic vesicles is released to intersynaptic space to interact with dopamine receptors in postsynaptic neurons. The clearance of dopamine from the synaptic clefts is mediated by a dopamine transporter that is localized in the plasma membrane of the dopaminergic neurons. Knockout animals for dopamine transporter present a dopamine deficiency and decreased vesicular storage of dopamine (Eriksen *et al.* 2010; Giros *et al.* 1996; Jones *et al.* 1998). The reuptake of dopamine from the synaptic clefts mediated by dopamine transporter is also a source of free cytosolic dopamine that can be stored in monoaminergic vesicles by the action of VMAT-2 (Fig. 1).

Dopamine degradation by monoamine oxidase

Dopamine excess in the cytosol is degraded by the action of the enzyme monoamine oxidase (MAO, E.C. 1.4.3.4) which catalyzes the oxidative deamination of the dopamine amino group to 3,4-dihydroxyphenylacetaldehyde with concomitant formation of ammonia and hydrogen peroxide. The product of this reaction is metabolized by aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid using NAD as electron donator (Fig. 2). The MAO enzymes are flavoenzymes containing FAD (Bach *et al.* 1988) where the isozymes labeled as A and B share 70% of primary structure identity. MAO enzymes are found at the outer membranes of mitochondria in neurons and glia cells (Weyler *et al.* 1990; Shih *et al.* 1997). MAO-B is expressed in serotonergic and histaminergic neurons as well as in astrocytes (Westlund *et al.* 1988; Saura *et al.* 1994) whereas monoamine oxidase type A (MAO-A) is primarily expressed in catecholaminergic neurons.

MAO-A has higher affinity for serotonin, dopamine, norepinephrine and epinephrine, whereas the substrates of MAO-B with high affinity include tyramine, phenylethylamine and MPTP (Shih 1991; Strolin-Benedetti *et al.* 1992; Cases *et al.* 1995). However, MAO-B also uses as substrates dopamine, serotonin and norepinephrine (Geha *et al.* 2002).

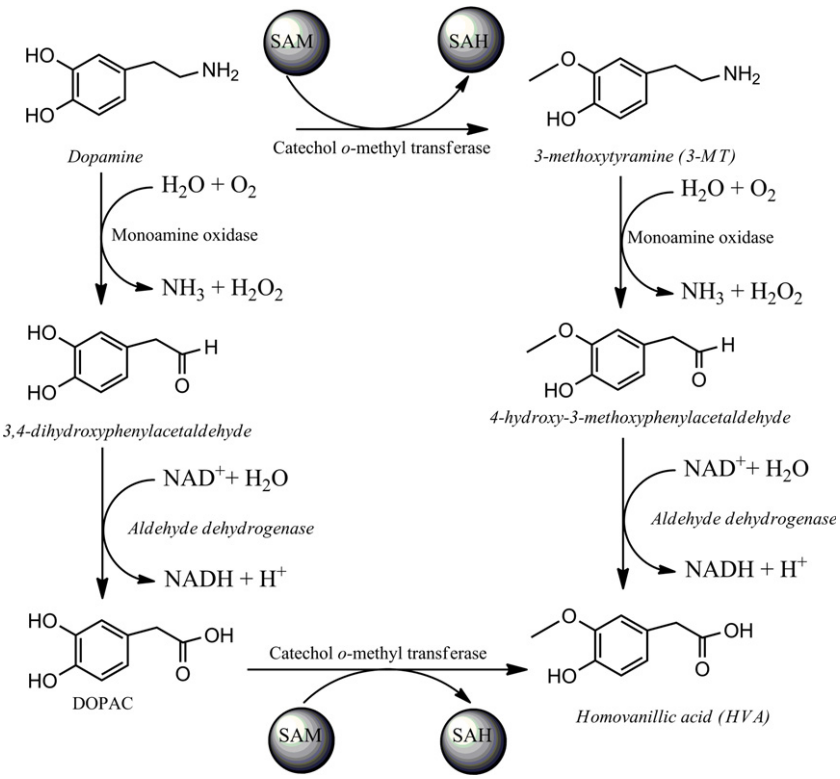

Fig. 2 Dopamine degradation catalyzed by monoamine oxidase and catechol methyl *ortho*-transferase.

MAO-A plays a role in maintenance of low concentrations of dopamine in the cytosol and it has been suggested that it plays a role in oxidative stress because the enzyme generates hydrogen peroxide and that it is a precursor of hydroxyl radicals. Rasagiline has been suggested to protect the brain in Parkinson's disease model systems by preventing the formation of reactive oxygen species catalyzed by MAO-B (Weinreb *et al.* 2011).

Dopamine degradation by catechol *ortho*-methyltransferase

Catechol *ortho*-methyltransferase (COMT; EC 2.1.1.6) also participates in dopamine degradation by catalyzing the methylation of dopamine to 3-methoxytyramine. Interestingly, MAO also catalyzes the oxidative deamination of 3-methoxytyramine to 3-methoxy-4-hydroxyphenylacetaldehyde. Another enzyme participating in dopamine degradation is aldehyde dehydrogenase that catalyzes the oxidation of 3-methoxy-4-hydroxyphenylacetaldehyde to homovanillic acid with concomitant formation of NADH. However, COMT also catalyzes the formation of homovanillic acid by methylating the metabolite formed during MAO-dependent dopamine degradation (see Fig. 2).

Both soluble (*S*-COMT) and membrane-bound (MB-COMT) isoforms have been reported in microglial and astroglial cells. COMT is also found in pyramidal neurons, cerebellar Purkinje, granular cells and striatal spiny neurons (Myöhänen *et al.* 2010). MB-COMT is localized to the cell body, axons and dendrites of rat cortical neurons.

The catalytic domain of COMT is localized in the C-terminal that is found in the extracellular space (Chen *et al.* 2011). Inhibitors of COMT such as entacapone have been used in combination with l-dopa to prolong its half-life (Marin and Obeso 2010).

Dopamine oxidation to ortho-quinones

Dopamine oxidation to dopamine *o*-quinone and its cyclization to aminochrome

It has been reported that dopamine is able to oxidize to *o*-quinones spontaneously in the absence of metal-ion catalysts under aerobic conditions (Linert *et al.* 1996). One electron oxidation of dopamine catalyzed by oxygen will generate dopamine *o*-semiquinone radical and a molecule of superoxide (Fig. 3, reaction 1). The formed dopamine *o*-semiquinone radical has two possibilities to react: (i) to disproportionate with a second dopamine *o*-semiquinone radical generating one molecule of dopamine *o*-quinone and one molecule of dopamine (Fig. 3, reaction 2); or (ii) to be one-electron oxidized to dopamine *o*-quinone by reducing oxygen to superoxide (Fig. 3, reaction 3). Several metal ions are able to catalyze dopamine oxidation such as manganese (III) both in aerobic and anaerobic conditions (Segura-Aguilar and Lind 1989); copper(II) (Paris *et al.* 2001); iron (III) (Paris *et al.* 2005a,b). Dopamine oxidation can also be catalyzed by enzymes such as xanthine oxidase, cytochrome P450, prostaglandin H synthase, lactoperoxidase and

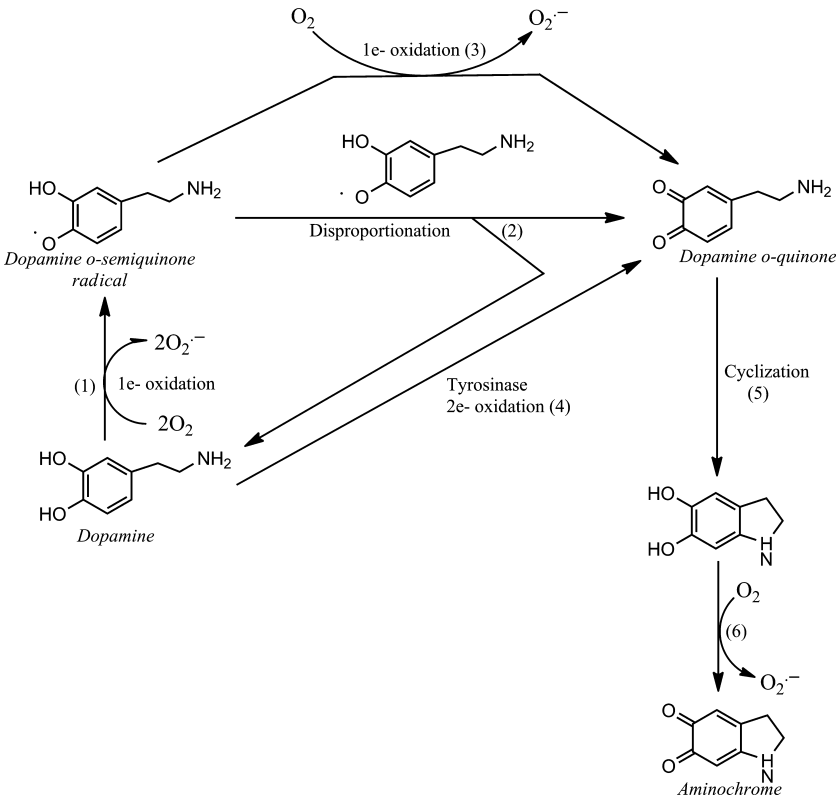

Fig. 3 Dopamine oxidation to aminochrome. Dopamine at physiological pH is able to oxidize to aminochrome in the presence of oxygen. Reducing oxygen to superoxide radical, dopamine can be one-electron oxidized to dopamine *o*-semiquinone radical (reaction 1), which is able to disportionate with another dopamine *o*-semiquinone radical and to generate one molecule of dopamine *o*-quinone and one molecule of dopamine (reaction 2). Alternatively, dopamine *o*-semiquinone radical can be one-electron oxidized to dopamine *o*-quinone by reducing oxygen to superoxide radical (reaction 3). Dopamine can also be two-electron oxidized to dopamine *o*-quinone, a reaction that it is catalyzed by tyrosinase (reaction 4). Dopamine *o*-quinone at physiological pH spontaneously cyclizes to leucoaminochrome that autoxidizes to form aminochrome, reducing oxygen to superoxide radical.

dopamine β-monooxygenase that use a peroxidase activity (Graham *et al*. 1978; Hastings 1995; Segura-Aguilar 1996; Foppoli *et al.* 1997; Galzigna *et al.* 2000; Thompson *et al.* 2000). Interestingly, the enzyme tyrosinase that plays an important role in the formation of pigments in the skin catalyzes the two-electron oxidation of dopamine to dopamine *o*-quinone (Fig. 3, reaction 4) without formation of dopamine *o*-semiquinone radical (Jimenez *et al.* 1984; Segura-Aguilar *et al.* 1998). Dopamine *o*-quinone is not stable at physiological pH (Segura-Aguilar and Lind 1989; Bisaglia *et al.* 2010) as the amino group of dopamine spontaneously rearranges and undergoes cyclization to generate leucoaminochrome (Fig. 3, reaction 5), which undergoes oxidation to form aminochrome (Fig. 3, reaction 6). Dopamine *o*-quinone has a yellow colour with an absorption maximum at 390 nm that can only be observed when dopamine oxidation is conducted below pH 2.0 (Segura-Aguilar and Lind 1989; Hawley *et al.* 1967; Harrison *et al.* 1968; Bisaglia *et al.* 2010). Dopamine *o*-quinone has been reported to form adducts with several proteins such as parkin (LaVoie *et al.* 2005), TH (Xu *et al.* 1998), mitochondrial glutathione peroxidase 4 (Hauser *et al.* 2013); dopamine transporter (Whitehead *et al.* 2001); quinoprotein adducts (Wang *et al.* 2011), mitochondrial complex I, III and V, isocitrate dehydrogenase, superoxide dismutase 2, JC-1, UCHL-1 (Van Laar *et al.* 2008, 2009); tryptophan hydroxylase (Kuhn and Arthur 1998). Dopamine *o*-quinone is a transient metabolite formed during dopamine oxidation to aminochrome at physiological pH and therefore, the question is whether the reports with dopamine oxidation can be assigned to dopamine *o*-quinone or the real responsible for this effect is aminochrome. The rate constant for dopamine *o*-quinone cyclization to aminochrome is only 0.15/s whereas the rate of aminochrome rearrangement to 5,6-dihydroxyindole is much slower (0.06/min) resulting in aminochrome accumulation (Tse *et al.* 1976; Bisaglia *et al.* 2010). The one dimensional NMR spectra of dopamine oxidation with $NaIO_4$ revealed the presence of dopamine, aminochrome and 5,6-dihydroxyindole but dopamine *o*-quinone was not observed because of the intramolecular cyclization of dopamine *o*-quinone is too rapid (Bisaglia *et al.* 2007). The question is which is the quinone species formed during dopamine oxidation that is relevant for the formation of protein adduct with alpha synuclein, parkin, mitochondrial glutathione peroxidase, dopamine transporter, mitochondria complex I, III and V and other proteins related to Parkinson's disease. The nucleophilic addition of glutathione to dopamine *o*-quinone is more rapid than dopamine *o*-quinone intramolecular cyclization because the constant rate of the nucleophilic addition of glutathione to dopamine *o*-quinone was estimated to be 200/s (Tse *et al.* 1976). Glutathione *S*-transferase M2 (GSTM2) catalyzed glutathione conjugation of dopamine *o*-quinone to 5-*S*-glutathionyl-dopamine the structure of which was determined by NMR (δ 6.77 and 6.87 ppm) and exhibited two peaks at 257 and 292 nm (Dagnino-Subiabre *et al.* 2000). The non-enzymatic

formation of 5-*S*-glutathionyldopamine was also determined by NMR when dopamine was oxidized in the presence of glutathione (Bisaglia *et al.* 2010). On the other side aminochrome conjugation with glutathione catalyzed by glutathione *S*-transferase M2 resulted in the formation of 4-*S*-glutathionyl-5,6-dihydroxyindoline determined with NMR.

Dopamine oxidation to aminochrome is dependent on the existence of free cytosolic dopamine; both VMAT-2 and MAO prevent dopamine oxidation by catalyzing dopamine uptake into monoaminergic vesicles and oxidative deamination. The presence of neuromelanin (NM) in the substantia nigra supports the existence *in vivo* of dopamine oxidation to aminochrome despite the presence of VMAT-2 and MAO, as aminochrome is the precursor of NM.

Aminochrome rearrangement to 5,6-indolequinone

The rearrangement of aminochrome to 5,6-dihydroxyindole (Fig. 4 reaction 2) and its oxidation to 5,6-indolequinone (Fig. 4, reaction 3) has been proposed based on *in vitro* experiments (Napolitano *et al.* 2011). The rate constant of aminochrome rearrangement to 5,6-dihydroxyindole is 0.06/min and the intramolecular cyclization has a rate constant of 0.15/s suggesting aminochrome accumulation (Bisaglia *et al.* 2010). The time evolution of dopamine oxidation determined by NMR revealed the presence of 5,6-indolequinone at 10 min or 40 min (Bisaglia *et al.* 2007) suggesting that the formation of 5,6-indolequinone is not an early process as 5,6-dihydroxyindole is the precursor of 5,6-indolequinone. The question is whether the rearrangement of aminochrome to 5,6-indolequinone is relevant under physiological conditions in the presence of enzymes such as flavoenzymes or conjugating enzymes such GSTM2 that catalyze rapid enzymatic reactions. This idea is supported by the fact that glutathione conjugation of aminochrome *in vitro* results in the formation of 4-*S*-glutathionyl-5,6-dihydroxyindoline (NMR δ 6.64 and 7.4 and absorption peaks at 277 and 295 nm; Segura-Aguilar *et al.* 1997) instead of 4-*S*-glutathionyl-5,6-dihydroxyindole (NMR δ 6.5 and 7.25 ppm and absorptions peaks at 295 and 397 nm; d'Ischia *et al.* 1987). However, a NMR study performed to determine the products of dopamine oxidation during the formation of adducts with alpha synuclein revealed the existence of aminochrome and alpha synuclein adducts at 4 min whereas 5,6-dihydroxyindole was detected at 40 min suggesting the role of aminochrome at the beginning and later 5,6-indolequinone in the formation of alpha synuclein adducts (Bisaglia *et al.* 2007).

Dopamine *o*-quinone, aminochrome or 5,6-indolequinone relevance in Parkinson's disease

Dopamine oxidation results in the formation of three quinones species during the formation of NM: dopamine *o*-quinone, aminochrome and 5,6-indolequinone. The question is which of these quinones play a role in the degenerative process of dopaminergic neurons containing NM. Dopamine *o*-quinone is the first *o*-quinone species formed under dopamine oxidation but the existence of this compound is very short because the constant rate of the intramolecular cyclization is very rapid (0.1/s) at physiological pH as this species is only stable at pH lower than 2.0 (Tse *et al.* 1976; Segura-Aguilar and Lind 1989). Interestingly, dopamine *o*-quinone reactivity is even faster with sulphydryl groups such as glutathione or the amino acid cysteine (Tse *et al.* 1976). There is no evidence of dopamine *o*-quinone presence in NMR spectra at physiological pH (Bisaglia *et al.* 2007). However, the enzymatic and non-enzymatic conjugation of

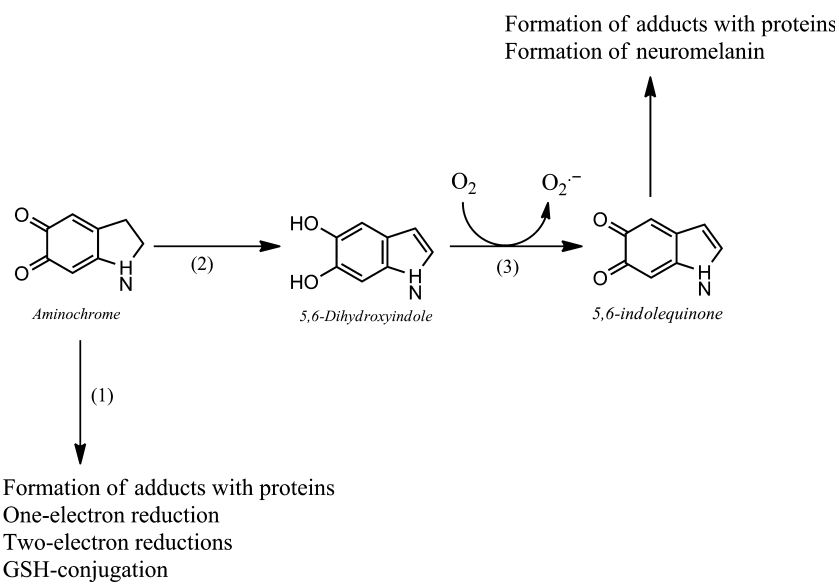

Fig. 4 Aminochrome rearrangement. Aminochrome formed in the cell is able to participate in different reactions such as formation of adducts with proteins, one-electron reduction, two-electron reduction and conjugation with glutathione (reaction 1). However, aminochrome can be rearranged by forming 5,6-dihydroxyindole (reaction 2) that can autoxidize to 5,6-indolequinone, reducing oxygen to superoxide radical (reaction 3). 5,6-Indolequinone is in equilibrium with quinone methide (reaction 4) that is also in equilibrium with quinonimine (reaction 5). 5,6-Indolequinone is able to form neuromelanin (NM) and adducts with proteins.

dopamine *o*-quinone with glutathione to 5-*S*-glutathionyl dopamine have been determined by NMR *in vitro* (Segura-Aguilar *et al.* 1997; Bisaglia *et al.* 2010) and 5-*S*-cysteinyldopamine that it is the degradation product of 5-*S*-glutathionyl dopamine that has been found in brain and cerebrospinal fluid of Parkinson's disease patients (Rosengren *et al.* 1985; Carstam *et al.* 1991; Cheng *et al.* 1996). This is a protective reaction that prevents neurotoxic effects of dopamine oxidation but the question is whether dopamine *o*-quinone is responsible for the generation of alpha synuclein neurotoxic protofibrils where this protein does not have a cysteine residue. Studies on the time evolution of dopamine oxidation products in the presence of alpha synuclein performed with NMR revealed the presence of stable alpha synuclein adducts when dopamine *o*-quinone is absent. The extremely rapid intramolecular cyclization of dopamine *o*-quinone does not seem to allow this quinone to form adducts with alpha synuclein as the alpha synuclein adducts appear at 4 min when aminochrome is present and alpha synuclein adducts grow with time where aminochrome disappears at 20 min. 5,6-dihydroxyindole is present at 40 min and alpha synuclein adducts continue to grow suggesting that aminochrome rearranges to 5,6-indolequinone (Bisaglia *et al.* 2007). However, the question is whether 5,6-indolequinone forms adducts with alpha synuclein or aminochrome adducts with alpha synuclein and undergo rearrangement to 5,6-indolequinone after the adduct formation. The short life of dopamine *o*-quinone opens the question on its role in the formation of adducts with other proteins.

The formation of NM is the result of dopamine oxidation after a chain of reactions that includes formation of dopamine *o*-quinone, aminochrome and 5,6-indolequinone. Both dopamine *o*-quinone and 5,6-indolequinone seem to be so unstable that it is not possible to record their NMR signals (Bisaglia *et al.* 2007; Pezzella *et al*. 2007). These quinones are likely to react with misfolded cross-β-sheets proteins which are shown to be present in the NM structure forming stable adducts (Engelen *et al.* 2012). Moreover, quinones can be detoxified by the large amount of cysteine and glutathione present forming soluble adducts (Fornstedt *et al.* 1986; Tse *et al.* 1976. Indeed, 5,6-indolequinone, the precursor of NM that has been proposed to be the most reactive species under dopamine oxidation (Bisaglia *et al.* 2007) could form adducts with alpha synuclein generating neurotoxic protofibrils/oligomers which seems to play a key role in Parkinson's disease (Bendor *et al.* 2013).

The instability of dopamine *o*-quinone and 5,6-indole quinone makes it difficult to study their role in the neurodegenerative process in Parkinson's disease. However, studies performed with aminochrome revealed that both DT-diaphorase (NQO1) and GSTM2 play an important protective role against aminochrome neurotoxicity both in dopaminergic neurons and astrocytes, respectively (Lozano *et al.* 2010; Huenchuguala *et al.* 2014).

Dopamine o-quinone, aminochrome and 5,6-indolequinone metabolism

Conversion to neuromelanin

As mentioned above, in dopaminergic neurons of the substantia nigra, dopamine oxidation, conjugation with other molecules and polymerization leads to the formation of NM, a dark pigment composed by a melanic structure bound to peptides and lipids. The synthesis of NM is driven by the excess of dopamine in the cytosol not accumulated by synaptic vesicles (Sulzer *et al.* 2000). Dopamine in the cytosol can be oxidized to dopamine *o*-quinone via iron-mediated catalysis and then can follow two different pathways: eumelanin or pheomelanin (Wakamatsu *et al.* 2003, 2012). The melanic portion of NM from human substantia nigra is composed of pheomelanin and eumelanin moieties with 1 : 3 ratio (Wakamatsu *et al.* 2003). Eumelanin is formed by oxidation of dopamine *o*-quinone with the formation of 5,6-dihydroxyindole. These precursors can then combine and form the polymer known as eumelanin. On the other hand, pheomelanin is formed when dopamine *o*-quinone reacts with l-cysteine leading to the formation of 2-S-cysteinyl-dopamine and 5-*S*-cysteinyl-dopamine. These two compounds are oxidized to form benzothiazines and polymerize to form pheomelanin portion of NM (Ito and Wakamatsu 2008). The reaction of dopamine *o*-quinone with cysteine seems to be faster than the cyclization of dopamine *o*-quinone to form aminochrome (Wakamatsu *et al.* 2012). This finding can be an explanation of the peculiar structure of NM that is composed by a pheomelanic core surrounded by eumelanin surface (Bush *et al.* 2006).

Although some steps in the formation of NM have been recently elucidated (Sulzer *et al.* 2000; Wakamatsu *et al.* 2003, 2012), it is still debated about which enzymes participate in the different steps of NM formation. In other melanins, the first steps of synthesis are catalyzed by tyrosinase, a metalloenzyme responsible for tyrosine hydroxylation and oxidation of catechol to quinone. Clear evidences of the presence of this enzyme in human substantia nigra are still lacking. Studies on mRNA have reported a small quantity of tyrosinase-coding mRNA (Xu *et al.* 1997; Greggio *et al.* 2005); however, mature protein was not detected in human substantia nigra by immunohistochemical staining and western blot analyses (Ikemoto *et al.* 1998; Tribl *et al.* 2007). Confirming the hypothesis that tyrosinase does not participate in the synthesis of NM, an old study reported the presence of NM in substantia nigra of albinos which do not have functional tyrosinase (Foley and Baxter 1958). The involvement of enzymes other than tyrosinase has been hypothesized: peroxidase (Galzigna *et al.* 2000), prostaglandin H synthase (Hastings 1995; Mattammal *et al.* 1995), xanthine oxidase (Foppoli *et al.* 1997) and TH (Haavik 1997). To date a neuronal specific enzymatic synthesis pathway has not been unequivocally demonstrated.

Another study suggested that the auto-oxidation of catechols to quinones with the subsequent addition of thiol groups occurs in the brain (Fornstedt *et al.* 1986).

It is also possible that proteins are incorporated into the melanic component during the synthesis of the pigment. This is suggested by *X*-ray diffraction studies that have shown a structural motif of 4.7 Å for isolated NM, whereas other natural and synthetic melanins showed a typical average 3.5 Å aromatic stacking interaction (Zecca *et al.* 2008a). The value for NM is similar to that observed in cross-β-sheets structure of amyloid protein aggregates (Makin and Serpell 2005). This is possibly because of the presence of a protein component forming the central core on which the dopamine polymerizes to form melanin component, with protein unfolding and aggregation, probably induced by dopamine *o*-quinone modification. This hypothesis is also supported by studies on protein–melanin interaction, in which it was shown that the polymerization of dopamine could be initiated by a dopamine *o*-quinone reaction with proteins (Nicolis *et al.* 2008; Ferrari *et al.* 2013).

NM has a peculiar structure, composed by a melanic polymer bound to peptides and lipids: all these components accumulate during ageing in specific pigmented organelles surrounded by a double membrane (Sulzer *et al.* 2008; Zecca *et al.* 2008a). As discussed above, NM has both pheomelanin and eumelanin components, where pheomelanin is present predominantly in the core and eumelanin on the surface (Bush *et al.* 2006; Ito 2006). The lipids are linked to the melanic component and are predominantly dolichols, with 14–22 isoprenic units (Zecca *et al.* 2000; Fedorow *et al.* 2005; Ward *et al.* 2007). The total amount of lipids covalently bound to NM represents about 18% of pigment weight (Engelen *et al.* 2012). On the other side, the amount of peptides comprises about 12–15% of NM weight (Zecca *et al.* 2000, 2008a; Engelen *et al.* 2012); these peptides derive from lysosomal, cytosolic and endoplasmic reticulum proteins (Tribl *et al.* 2005), although a more recent and reliable characterization of lipids and proteins contained in NM organelles has been performed (Zucca *et al.* 2014).

Formation of adducts with proteins

Aminochrome is able to form adducts with proteins resulting in the inactivation of physiological pathways. Aminochrome promotes the formation and stabilization of neurotoxic protofibrils of alpha synuclein (Conway *et al.* 2001; Norris *et al.* 2005). The formation of aminochrome adducts with alpha synuclein is followed by the formation of 5,6-indolequinone adducts with alpha synuclein (Bisaglia *et al.* 2007). It has also been reported that dopamine *o*-quinone is able to form adducts with complex I, III and V of electron transport chain and isocitrate dehydrogenase of mitochondria (Van Laar *et al.* 2009). Parkin, an ubiquitin ligase of proteasome system, is inactivated as a consequence of adduct formation with aminochrome (LaVoie *et al.* 2005). Ubiquitin carboxyl-terminal hydrolase isozyme L1, another protein associated to familiar Parkinson′s disease, also reacts with aminochrome (Van Laar *et al.* 2009). Furthermore, aminochrome induces the disruption of cytoskeleton architecture by forming adducts with actin and α- and β-tubulin (Paris *et al.* 2010). Finally, the inactivation of human dopamine transporter, tyrosine and tryptophan hydroxylase has been reported (Kuhn and Arthur 1998; Xu *et al.* 1998; Whitehead *et al.* 2001). Recently, it has been reported that dopamine *o*-quinone forms adduct with mitochondrial glutathione peroxidase 4 (Hauser *et al.* 2013).

One-electron reduction of aminochrome

Aminochrome is an acceptor of electrons from flavoenzymes that use NADH or NADPH as electron donator and it can be one-electron reduced to leucoaminochrome-*o*-semiquinone radical that it is tremendously reactive with oxygen (Baez *et al.* 1995; Segura-Aguilar *et al.* 1998). Leucoaminochrome-*o*-semiquinone radical reduces oxygen to superoxide radical with concomitant autoxidation to aminochrome, generating a redox cycling between aminochrome and the leucoaminochrome *o*-semiquinone radical. This redox cycling continues until NADH and oxygen are depleted, generating an energy collapse as NADH and oxygen are not available for ATP synthesis in mitochondria; moreover, the formation of superoxide radicals contributes to oxidative stress, as they are precursors of hydroxyl radicals. The oxidative stress is potentiated by the prevalence of oxidized glutathione as a consequence of the depletion of NADPH, used by flavoenzymes as electron donator. The reduction of aminochrome by one-electron to leucoaminochrome-*o*-semiquinone radical has been reported to be a neurotoxic reaction (Paris *et al.* 2001, 2005a,b, 2009, 2010, 2011; Arriagada *et al.* 2004 and Fuentes *et al.* 2007; Díaz-Véliz *et al.* 2008; Muñoz *et al.* 2012).

Two-electron reduction of aminochrome

All the flavoenzymes with the exception of DT-diaphorase (EC.1.6.99.2) catalyze one-electron reduction of aminochrome. DT-diaphorase is a flavoenzyme containing two FAD molecules that catalyze the two-electron transfer to quinones using both NADH and NADPH as electron donator. This enzyme is found in the majority of the organs including brain, localized in different regions such as striatum, substantia nigra, frontal cortex, hippocampus, cerebellum and hypothalamus. DT-diaphorase is expressed not only in dopaminergic neurons and astrocytes of both substantia nigra and ventral tegmental area, but also in Bergmann glia, astrocytes and tanycytes. Interestingly, DT-diaphorase is responsible for the 97% of total quinone reductase activity in substantia nigra (Schultzberg *et al.* 1988). Aminochrome is two-electron reduced by DT-diaphorase to leucoaminochrome (Segura-Aguilar and Lind 1989) that autoxidizes in the presence of superoxide radicals;

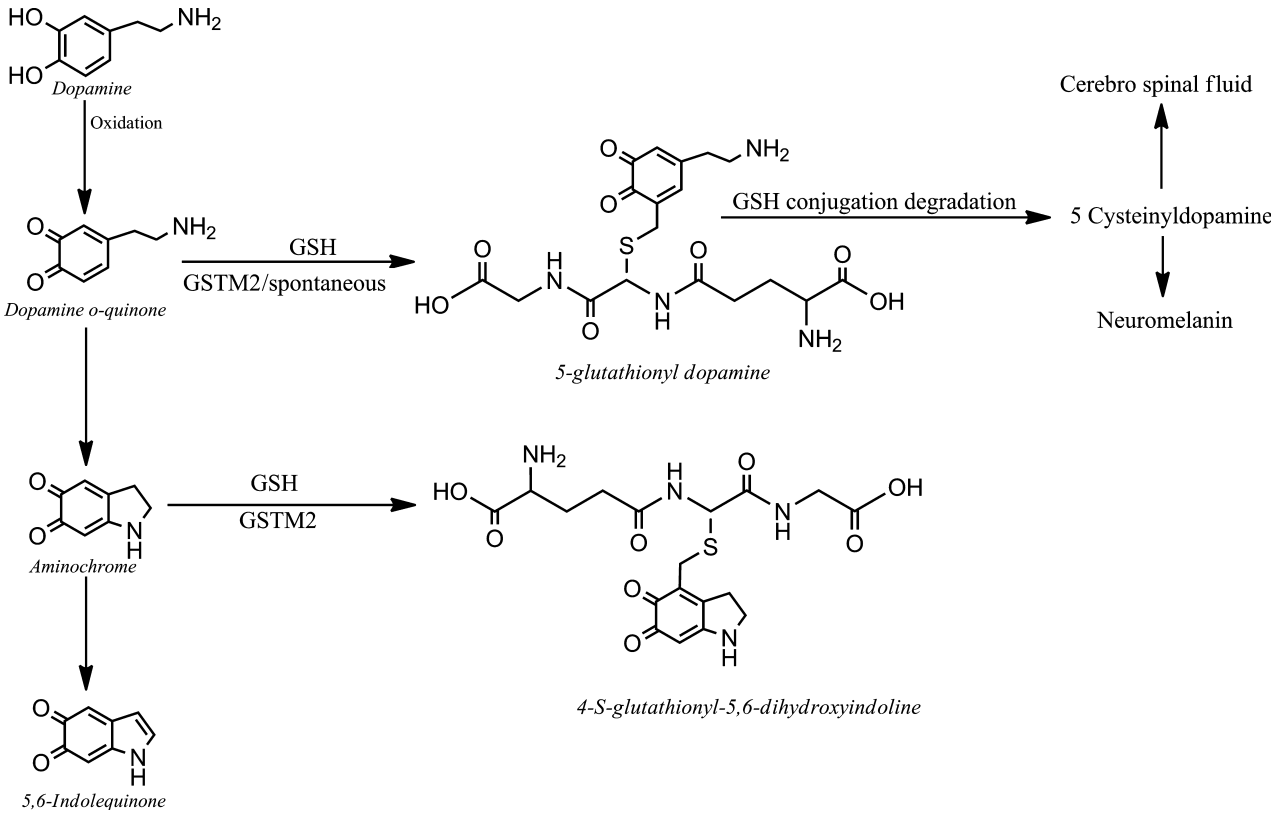

Fig. 5 Dopamine *o*-quinone and aminochrome conjugation with glutathione. Dopamine *o*-quinone can be conjugated with glutathione spontaneously both in dopaminergic neurons and astrocytes. However, dopamine *o*-quinone can be conjugated by GSTM2 to 5-glutathionyl dopamine in astrocytes that under the glutathione conjugation degradation form, 5-*S*-cysteinyl dopamine has been found in human cerebrospinal fluid and neuromelanin (NM). In astrocytes GSTM2 catalyzes aminochrome conjugation to 4-*S*-glutathionyl-5,6-dihidroxiindoline.

however, its autoxidation is prevented by superoxide dismutase present in the cytosol (Baez *et al.* 1995). DT-diaphorase has been proposed to be a neuroprotective enzyme preventing aminochrome neurotoxicity. Studies where DT-diaphorase has been inhibited by dicoumarol or its expression was strongly decreased using a siRNA support the protective role of the enzyme by preventing aminochrome neurotoxicity both in cell cultures (Arriagada *et al.* 2004; Lozano *et al.* 2010; Paris *et al.* 2010, 2011; Muñoz *et al.* 2012) or in experiments *in vivo* (Segura-Aguilar *et al.* 2002; Díaz-Véliz *et al.* 2002, 2004a,b, 2008).

Glutathione conjugation of dopamine *o*-quinone and aminochrome

An important detoxification mechanism of potentially toxic molecules is glutathione conjugation. It has been reported that aminochrome can be conjugated with glutathione to 4-*S*-glutathionyl-5,6-dihydroxyindoline and GSTM2 is the most active isoform (Fig 5). Interestingly, the conjugate 4-*S*-glutathionyl-5,6-dihydroxyindoline is stable in the presence of oxygen, hydrogen peroxide and superoxide radicals, suggesting that this is a final product for elimination (Baez *et al.* 1997; Segura-Aguilar *et al.* 1997). GSTM2 catalyzes also the conjugation of the precursor of aminochrome dopamine *o*-quinone to 5-*S*-glutathionyldopamine (Dagnino-Subiabre *et al.* 2000; Fig. 5) that it is degraded to 5-*S*-cysteinyldopamine (Shen *et al.* 1996) as glutathione is a tripeptide composed of γ-L-Glu-L-Cys-Gly that undergo enzymatic degradation to 5-*S*-cysteinyldopamine. 5-*S*-cysteinyldopamine was found to be present in substantia nigra, putamen, caudate nucleus and globus pallidus, NM and the cerebrospinal fluid of Parkinson's disease patients (Rosengren *et al.* 1985; Carstam *et al.* 1991; Cheng *et al.* 1996). The conjugation of aminochrome and dopamine *o*-quinone has been proposed to be a protective mechanism against aminochrome neurotoxicity in astrocytes. In dopaminergic neurons, the spontaneous addition of glutathione to dopamine *o*-quinone is possible because of the high reactivity of this *o*-quinone with glutathione (Tse *et al.* 1976). GSTM2 is not expressed in dopaminergic neurons and glutathione conjugation of aminochrome can proceed spontaneously or with other glutathione *S*-transferases but the rate of these reactions are significant slower.

Dopamine o-quinone, aminochrome, 5,6-indolequinone and neuromelanin in Parkinson's disease

Parkinson's disease and dopamine *o*-quinone, aminochrome and 5,6-indolequinone

The degeneration of dopaminergic neurons containing NM resulting in the presentation of motor symptoms in Parkinson's disease seems to be preceded by a pre-symptomatic phase with olfactory disturbances, sleep fragmentation and depression (Wolters and Braak 2006). Six stages have been proposed (Braak *et al.* 2004) where the disease start in the olfactory bulb, motor component of cranial nerve X and enteric plexus (stage 1). The disease progresses to the caudal raphe, locus coeruleus and magnocellular reticular formation (stage 2). The substantia nigra, amygdala central subnucleus, Meynert's nucleus and pedunculopontine tegmental nucleus are affected around 5 years before the appearance of the motor symptoms (stage 3) whereas the prefrontal cortex and tertiary sensory association areas are affected after 10 years of appearance of the motor symptoms (stage 5). Finally, the secondary and then the primary motor and sensory areas are affected (stage 6) (Hawkes *et al.* 2010).

The molecular mechanism responsible for the loss of dopaminergic neurons containing NM is still unknown despite the enormous effort in basic research. The discovery of genes associated with familial form of Parkinson′s disease gave a potent impulse in Parkinson′s disease basic research. Several genes were found to be associated with familial Parkinson′s disease such as alpha-synuclein (Polymeropoulos *et al.* 1997); parkin (Hattori *et al.* 1998; Kitada *et al.* 1998; Abbas *et al.* 1999); LRRK-2 (Kachergus *et al.* 2005); PINK1 (Valente *et al.* 2004); DJ-1 (Bonifati *et al.* 2003); ATP13A2 (Ramirez *et al.* 2006), etc. Although these mutations are rare and cannot explain the sporadic Parkinson′s disease, the study of these proteins' function has yielded valuable information that can be useful in understanding their role in the disease. Today, there is a general agreement in the scientific community that the molecular mechanism responsible for the degeneration of NM-containing dopaminergic neurons in Parkinson′s disease involves mitochondria dysfunction; protein degradation dysfunction; aggregation of alpha synuclein to neurotoxic protofibrils; oxidative stress and neuroinflammation (Block *et al.* 2007; Zecca *et al.* 2008c; Ebrahimi-Fakhari *et al.* 2012; Exner *et al.* 2012; Rohn 2012; Hauser and Hastings 2013; Kalia *et al.* 2013; Merad-Boudia *et al*. 1998; Martinez-Vicente and Vila 2013; Mullin and Schapira 2013; Subramaniam and Chesselet 2013; Taylor *et al.* 2013). The question is to find the neurotoxin linking all these mechanisms, and as we discussed before dopamine oxidation generates dopamine *o*-quinone, aminochrome and 5,6-indolequinone during NM formation. Interestingly, these quinones are directly involved in four of the five mechanisms proposed to be related in the neurodegenerative process in Parkinson′s disease as (i) aminochrome induces and stabilize the formation of neurotoxic protofibrils of alpha synuclein (Conway *et al.* 2001; Norris *et al.* 2005; Fig. 6, reaction 7). Aminochrome forms adducts with alpha synuclein in the motif 125YEMPS129 (Norris *et al.* 2005; Dibenedetto *et al.* 2013). 5,6-Indolequinone is also involved in the formation of adduct with alpha synuclein (Bisaglia *et al.* 2007). The formation of neurotoxic alpha synuclein protofibrils is dependent on specific mutation such as Ala30Pro, Glu46Lys, His50Gln, Gly51Asp and Ala53Thr in familial Parkinson′s disease (Trinh and Farrer 2013) whereas in the sporadic Parkinson′s disease, aminochrome/5,6-indolequinone may be the responsible for the formation of neurotoxic protofibrils in specific dopaminergic neurons containing NM. Dopamine *o*-quinone, aminochrome and 5,6-indolequinone are formed in the NM-containing dopaminergic neurons as they are the precursors of NM that accumulates with age (Zecca *et al.* 2002); (ii) dopamine *o*-quinone induces mitochondria dysfunction by forming adducts that inactivate complex I and III of electron transport chain and complex V (Fig. 6, reaction 8), responsible of oxidative phosphorylation of ADP to ATP in mitochondria (Van Laar *et al.* 2009). Dopamine *o*-quinones also form adducts with isocitrate dehydrogenase (Van Laar *et al.* 2009) that is a component of Krebs cycle that plays an important role in the oxidative catabolism of glucose to ATP by generating NADH and $FADH_2$ required to produce ATP in the mitochondria. Depletion of NADH is also induced by one-electron reduction of aminochrome to leucoaminochrome *o*-semiquinone radical that generates a redox cycling between aminochrome and leucoaminochrome *o*-semiquinone, which is extremely reactive with oxygen (Baez *et al.* 1995; Segura-Aguilar *et al.* 1998). DJ-1 protein has been associated with a familial form Parkinson′s disease (Bonifati *et al.* 2003) and it has been proposed to play a protective role in the mitochondria by reducing oxidative stress generated during the inhibition of the electron transport chain (Canet-Avilés *et al.* 2004; Blackinton *et al.* 2009; Trempe and Fon 2013). Mitochondria from DJ-1 deficient animals generate more reactive oxygen species in comparison with control animals and DJ-1 deficiency leads to altered autophagy in cell cultures (Irrcher *et al.* 2010). Interestingly, dopamine *o*-quinone forms adducts with DJ-1 protein (Van Laar *et al.* 2009; Fig. 6 reaction 9) preventing the protective role against oxidative stress in the mitochondria when the electron transport is inhibited. Aminochrome also disturbs mitochondria movements along the axons and dendrites as it forms adducts with actin and α- and β-tubulin generating abnormal aggregates (Paris *et al.* 2010; Fig. 6, reactions 10 and 11, respectively) which prevent the formation of microfilaments and microtubules required for axonal and dendrite transport of mitochondria (Ligon and Steward 2000); (iii) the degradation of proteins mediated by proteasome and lysosomal systems is an essential cellular event for proteins turnover

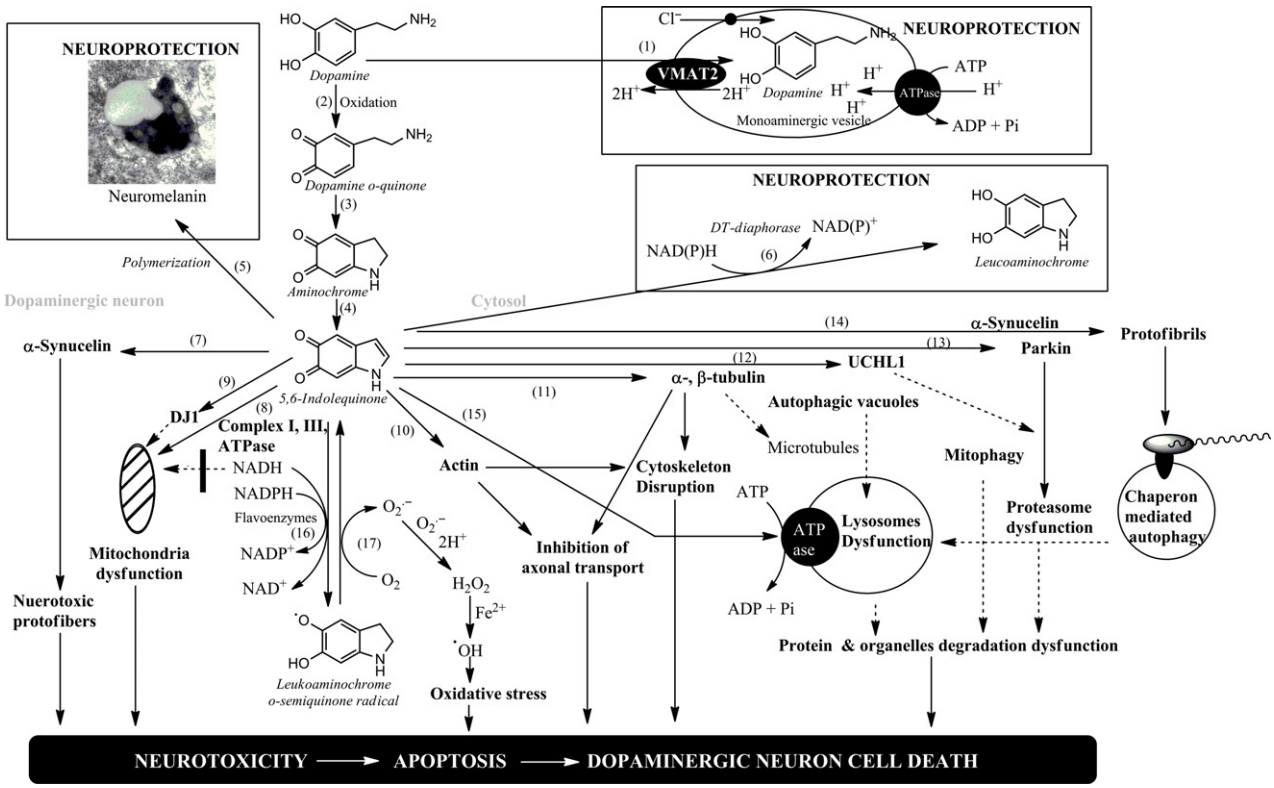

Fig. 6 The possible effects of dopamine *o*-quinone, aminochrome and 5,6-indolequinone during dopamine oxidation in dopaminergic neurons. Dopamine oxidation generates dopamine *o*-quinone, aminochrome and 5,6-indolequinone during neuromelanin (NM) formation. Dopamine *o*-quinone and 5,6-indolequinone are too unstable to separate their role from aminochrome and therefore, we put these 3 *o*-quinones in the same pathway. Dopamine *o*-quinone, aminochrome and 5,6-indolequinone metabolism can be divided in neuroprotective and neurotoxic reactions. In the neuroprotective reactions we have dopamine uptake into monoaminergic vesicles mediated by vesicular monoaminergic transporter-2 (reaction 1) that prevent dopamine oxidation. Dopamine oxidizes to dopamine *o*-quinone (reaction 2) that undergoes intramolecular cyclization to aminochrome (reaction 3) and aminochrome structure is rearranged to 5,6-indolequinone (reaction 4) before it polymerizes to form NM (reaction 5). Aminochrome can be two-electron reduced to leucoaminochrome catalyzed by DT-diaphorase (reaction 6) preventing aminochrome participation in neurotoxic reactions. The neurotoxic reactions where dopamine *o*-quinone, aminochrome and 5,6-indolequinone can participate include the formation of adducts with alpha synuclein to neurotoxic protofibrils where both aminochrome and 5,6-indolequinone play a role (reaction 7); the formation of adduct of dopamine *o*-quinone with complex I, III and V (ATPase; reaction 8) of electron transport chain and oxidative phosphorylation and isocitrate dehydrogenase of Krebs cycle that induces mitochondria dysfunction; the formation of adducts dopamine *o*-quinone and inactivation of DJ-1 protein (reaction 9) under normal conditions prevents oxidative stress generated by inhibition of mitochondrial electron transport; the one-electron reduction of aminochrome to leucoaminochrome *o*-semiquinone radical (reaction 16) immediately autoxidizes reducing oxygen to superoxide radicals (reaction 17) and the latter spontaneously or enzymatically catalyzes the formation of hydrogen peroxide which is the precursor of hydroxyl radicals generating oxidative stress. The reaction 16 and 17 generates a redox cycling that depletes NADH, NADPH and O_2. Aminochrome also forms adducts with actin (reaction 10) that disrupt cytoskeleton architecture and prevent the formation of actin neurofilament required for mitochondria transport in the axons and dentrites; the formation of adducts with α- and β-tubulin (reaction 11) that prevent the formation of microtubules required for axonal transport and the fusion of autophagy vacuoles with lysosomes; Aminochrome also induces lysosome dysfunction probably by forming adducts with a vacuolar ATPase (reaction 15) that maintain low pH inside the lysosomes. Both the lysosome dysfunction and the prevention of the fusion of autophagy vacuoles with lysosomes result in the inhibition of lysosomal degradation of proteins and organelles. The formation of protofibrils induced by aminochrome/5,6-indolequinone also inhibits chaperon mediated autophagy; dopamine *o*-quinone forms adducts and inactivate UCHL-1 (reaction 12). UCHL-1 plays a key role in ubiquitin proteasome system by recycling ubiquitin monomers for re-use in the proteasome (Healy *et al.* 2004); dopamine *o*-quinone forms adduct and inactivate parkin that is an ubiquitine ligase 3 of proteasome (reaction 13). Inactivation of both ubiquitin proteasome system and autophagy-lysosome system result in protein and organelles degradation dysfunction. Alpha synuclein protofibrils inhibit chaperon-mediated autophagy (reaction 14).

and damaged proteins clearance. Macroautophagy also plays an important role to recycle damaged organelles such as mitochondria. Aminochrome has been reported to induce dysfunction of proteasome system (Zafar *et al.* 2006; Zhou and Lim 2009) as a consequence of the inactivation of parkin induced by dopamine *o*-quinone, that it is an ubiquitin ligase

3 of proteasome system (LaVoie *et al.* 2005; Fig 6, reaction 13). Dopamine *o*-quinone also forms adducts and inactivates UCHL-1 that plays an important role in ubiquitin proteasome system by hydrolyzing the peptide-ubiquitin bonds and recycle ubiquitin monomers for re-use in the same process (Healy *et al.* 2004; Fig. 5 reaction 12). Moreover, aminochrome inhibits autophagy by preventing the fusion of autophagy vacuoles with lysosomes mediated by microtubules because of aminochrome ability to forms adducts with α- and β-tubulin that are required for microtubules formation (Paris *et al.* 2010; Fig. 6, reaction 11). Aminochrome induces lysosome dysfunction by affecting lysosome acidification that it is essential for protein degradation in lysosomes (Huenchuguala *et al.* 2014; Fig. 6, reaction 15). Finally, the formation of adducts of aminochrome with alpha synuclein inhibit chaperon-mediated autophagy (Cuervo *et al.* 2004); (iv) aminochrome induces oxidative stress when flavoenzymes catalyze its one-electron reduction to leucoaminochrome *o*-semiquinone radical (Fig. 6, reaction 16) that immediately autoxidizes in the presence of oxygen generating superoxide radical and aminochrome (Baez *et al.* 1995; Segura-Aguilar *et al.* 1998; Arriagada *et al.* 2004; Fig 6, reaction 17). The flavoenzymes using NADH or NADPH reduce aminochrome again to leucoaminochrome *o*-semiquinone radical that autoxidize again, generating a redox cycle (Fig. 6, reaction 16 and 17) which continues until O_2 and NADH or NADPH are depleted. The superoxide radicals dismutate generating hydrogen peroxide that is the precursor of hydroxyl radicals, potentiating oxidative stress.

Neuromelanin and Parkinson's disease: protective and toxic role

NM accumulates as a function of age in the human substantia nigra, while in Parkinson's disease patients NM concentrations decreased up to 50–60% compared to age-matched controls, as a result of the loss of neurons containing NM (Zecca *et al.* 2002). Indeed, Parkinson's disease is characterized by preferential loss of those dopaminergic neurons that contain NM (Marsden 1983; Hirsch *et al.* 1988; Gibb and Lees 1991; Kastner *et al.* 1992). Thus, several studies in the last 20 years have tried to identify the role of NM in the substantia nigra both in physiological conditions and in the pathogenesis of Parkinson's disease.

The role of NM is still debated and it appears to be two-faced and depending on the cellular conditions. First of all the synthesis of NM is a protective mechanism against dopamine toxicity. As mentioned above, it was shown by *in vitro* experiments that an excess of cytosolic dopamine lead to the formation of NM and that an over-expression of VMAT-2 is able to reduce the synthesis of the pigment (Sulzer *et al.* 2000). These results suggest that the synthesis of NM converts potentially toxic quinones and semiquinones in a more stable and inactive polymer, thus preventing redox reactions which damage neurons. Quinones are highly reactive and can modify proteins which undergo structural changes (LaVoie *et al.* 2005). The resulting structure could be the initial step in the formation of toxic and insoluble fibrils, similar to that observed for α-synuclein (Sulzer and Zecca 2000; Conway *et al.* 2001). Another potential harmful pathway of quinones is the inhibition of NADH reductase in mitochondria (Li and Dryhurst 1997). It was also observed that oxidized dopamine, and also oxidized 3,4-dihydroxyphenylacetic acid, can inhibit complex I and complex II activities in a dose-dependent manner (Gautam and Zeevalk 2011). As discussed above the physiological conversion of dopamine to 3,4-dihydroxyphenylacetaldehyde by MAO leads also to the formation of hydrogen peroxide, which can react with metals, generating hydroxyl radicals via Fenton's reaction (Stokes *et al.* 1999). It should also be noted that the metabolite 3,4-dihydroxyphenylacetaldehyde can be toxic for neurons via different pathways (Goldstein *et al.* 2013).

Another potentially protective feature of NM resides in its ability to sequester metals including iron, copper and zinc (Zecca *et al.* 1994, 2001a, 2008a) in a redox inactive form (Shima *et al.* 1997). Iron is the most abundant metal in NM and it seems that the pigment is the principal iron storage site in substantia nigra dopaminergic neurons, thus playing a crucial role in iron homeostasis (Zecca *et al.* 2001b, 2004, 2008b). Iron is bound at two different sites, defined as high and low affinity sites depending on their binding strength (Double *et al.* 2003; Fasano *et al.* 2006; Zecca *et al.* 2008b) where most of the iron is bound to high affinity sites in an inactive form preventing reactive oxygen species (ROS) production via Fenton's reaction (Zecca *et al.* 2008b). Other metals sequestered into NM are aluminum, lead, manganese, mercury, cobalt, cadmium, and selenium (Zecca *et al.* 1994, 2001a, 2008a; Bohic *et al.* 2008). Since environmental exposure to these metals seems to be linked to an increase in the incidence of Parkinson's disease (Gorell *et al.* 1999), NM could have a protective role by sequestering these potentially toxic metals. In particular, a remarkable accumulation of lead was observed in NM of substantia nigra and this is notably since an increased risk for Parkinson's disease has been associated to occupational exposure to lead (Coon *et al.* 2006).

NM can have a protective role also by binding toxic molecules known to induce Parkinson's disease-like symptoms, such as paraquat (Lindquist *et al.* 1988) and MPP^+, the toxic metabolite of MPTP (D'Amato *et al.* 1986). Some dopaminergic drugs are also sequestered by NM, such as chlorpromazine, haloperidol and imipramine (Salazar *et al.* 1978; Larsson 1993). On the other side, it should be noted that NM may gradually release these compounds into the cytosol under certain circumstances, losing its protective role (Lindquist *et al.* 1987; Karlsson and Lindquist 2013).

However, in particular conditions, NM can become potentially toxic for neurons. For instance, under iron

overload conditions, as may occur in Parkinson's disease (Earle 1968; Dexter *et al.* 1987, 1989; Sofic *et al.* 1988), NM can bind iron also in the low affinity sites where the metal is in a reactive form and could promote noxious redox reactions (Zareba *et al.* 1995; Double *et al.* 2002; Zecca *et al.* 2008b). Indeed, an increased content of redox-active iron has been associated with NM in substantia nigra of Parkinson's disease patients (Good *et al.* 1992; Jellinger *et al.* 1992; Faucheux *et al.* 2003). NM saturated with iron may also catalyze the oxidation of dopamine, thus enhancing oxidative damage of proteins (Zecca *et al.* 2008b).

Some authors proposed that NM could be toxic for neurons via the inhibition of ubiquitin proteasome system, with consequent accumulation of ubiquitinated and denatured proteins in the cytoplasm (Shamoto-Nagai *et al.* 2004). The same group has also suggested that NM could have a direct effect on mitochondria, releasing redox active iron thus promoting the production of ROS and reactive nitrogen species (Shamoto-Nagai *et al.* 2006). Despite these intriguing results, these two *in vitro* experiments only consider the direct effect of NM on ubiquitin proteasome system and mitochondria: it should be considered that NM is physiologically enveloped into organelles and a direct interaction with these cellular systems may occur only in special circumstances.

Furthermore, NM could be involved in exacerbating the neuroinflammatory and neurodegenerative processes of Parkinson's disease. NM is released from dying neurons in the substantia nigra of parkinsonian subjects and sustained microglial activation has been observed along with the presence of NM in extracellular space (McGeer *et al.* 1988; Banati *et al.* 1998; Langston *et al.* 1999). In the extracellular milieu of the substantia nigra, NM could release metals and toxic compounds previously accumulated further triggering neuronal damage. Moreover, when extracellular NM particles are phagocytosed and degraded by activated microglia, toxic compounds and redox active metals efficiently immobilized into NM could be released, exacerbating neuronal damage and microglial activation. The pigment could also be degraded in presence of hydrogen peroxide resulting in the exposure of its pheomelanic core, which has an oxidation potential thermodynamically favourable for generating oxidative stress (Bush *et al.* 2006). In addition, *in vitro* experiments have shown that human NM is able to induce chemotaxis in microglia cultures. Microglia cells actively phagocytose NM and release not only neurotoxic mediators such as tumournecrosis factor α, interleukin 6 and nitric oxide (Wilms *et al.* 2003), but also reactive species like superoxide and hydrogen peroxide (Zhang *et al.* 2011, 2013). In co-cultures microglia/neurons treated with human NM, activation of microglia and neuronal death occur; the toxic effect of extracellular NM seems to be mediated by microglia, as in the absence of microglia NM does not seem to be toxic for neurons. In the neurodegeneration produced by microglia activated by NM the involvement of macrophage antigen complex-1 and phagocytic oxidase of microglia has been demonstrated (Zhang *et al.* 2011, 2013). *In vivo* experiments have shown that NM injected into rat substantia nigra induces microglia activation and degeneration of dopaminergic neurons (Zecca *et al.* 2008c; Zhang *et al.* 2011).

In summary, NM has both protective and toxic role. In physiological conditions NM protects substantia nigra neurons from the noxious effects of dopamine and its metabolites, metals and other compounds. However, when there is an iron overload NM could participate in neurodegeneration by exacerbating oxidative stress. Finally, in case of neuronal death NM is released in the extracellular space and could contribute to neuronal damage by inducing microglia activation.

Conclusions

Dopamine oxidation seems to be a complex mechanism where dopamine *o*-quinone is formed and immediately undergoes intramolecular cyclization to aminochrome at physiological pH. Aminochrome structure rearranges to 5,6-indolequinone as the precursor of NM. Both dopamine *o*-quinone and 5,6-indolequinone are so unstable that it is difficult to separate their roles in dopamine oxidation toxicity from aminochrome. These *o*-quinones seem to play an important role in the loss of NM-containing dopaminergic neurons in Parkinson′s disease. Dopamine *o*-quinone, aminochrome and 5,6-indolequinone are directly involved in four mechanisms that seem to play a role in Parkinson′s disease such as mitochondria dysfunction, formation and stabilization of alpha synuclein protofibrils, protein degradation dysfunction (proteasomal and lysosomal systems) and oxidative stress. Dopamine *o*-quinone, aminochrome and 5,6-indolequinone are able to form adducts and inactivate several proteins that have been found to be associated with familial form of Parkinson′s disease such as alpha synuclein, parkin, DJ-1 and UCHL-1. Dopamine *o*-quinone, aminochrome and 5,6-indolequinone are neurotoxic when they (i) form adducts with proteins; and (ii) aminochrome one-electron reduced to leucoaminochrome *o*-semiquinone radical that it is extremely reactive with oxygen generating a redox cycling that depletes oxygen and NADH or NADPH. DT-diaphorase catalyzes the two-electron reduction of aminochrome and prevents all aminochrome neurotoxic reactions. Other protective reactions are dopamine uptake by VMAT-2 that completely prevents aminochrome formation. In addition, VMAT-2 is also able to take up aminochrome to monoaminergic vesicles preventing aminochrome's participation in neurotoxic reactions (Muñoz *et al.* 2012). Aminochrome polymerization to NM is also a protective reaction that prevents aminochrome neurotoxicity. This pigment is found to accumulate during ageing in lysosomal organelles in

substantia nigra neurons and locus coeruleus. NM can play a protective role even by chelating redox/toxic metals (Fe, Cu, Pb, Cd, Hg) to form stable complexes. However, NM released by dying neurons in the extracellular space can activate microglia which in turn can induce neurodegeneration by targeting neuronal processes and the release of reactive species and proinflammatory molecules. Thus, depending on the cellular context, NM can play either a protective role or a toxic one.

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

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