Neurotoxicity Research (2019) 36:746–755
https://doi.org/10.1007/s12640-019-00078-y

ORIGINAL ARTICLE

Superoxide Dismutases SOD1 and SOD2 Rescue the Toxic Effect of Dopamine-Derived Products in Human SH-SY5Y Neuroblastoma Cells

Alice Biosa[1] · Federica De Lazzari[1] · Anna Masato[1] · Roberta Filograna[1,2] · Nicoletta Plotegher[1] · Mariano Beltramini[1] · Luigi Bubacco[1] · Marco Bisaglia[1]

Received: 1 April 2019 / Revised: 12 June 2019 / Accepted: 14 June 2019 / Published online: 21 June 2019

Abstract

The preferential loss of dopaminergic neurons in the substantia nigra pars compacta is one of the pathological hallmarks characterizing Parkinson's disease. Although the pathogenesis of this disorder is not fully understood, oxidative stress plays a central role in the onset and/or progression of Parkinson's disease and dopamine itself has been suggested to participate in the preferential neuronal degeneration through the induction of oxidative conditions. In fact, the accumulation of dopamine into the cytosol can lead to the formation of reactive oxygen species as well as highly reactive dopamine-quinones. In the present work, we first analyzed the cellular damage induced by the addition of dopamine (DA) in the culture medium of SH-SY5Y cells, discriminating whether the harmful effects were related to the generation of reactive oxygen species or to the toxicity associated to dopamine-derived quinones. Then, we tested and demonstrated the capability of the antioxidant enzymes SOD1 and SOD2 to protect cells from the noxious effects induced by DA treatment. Our results support further exploration of superoxide dismutating molecules as a therapeutic strategy against Parkinson's disease.

Keywords Dopamine · Dopamine quinones · Oxidative stress · Parkinson's disease · Superoxide dismutation

The preferential loss of dopaminergic neurons in the substantia nigra pars compacta is one of the pathological hallmarks of Parkinson's disease (PD)(Forno 1996). The loss of these neurons is responsible for the motor dysfunctions associated to the disorder, which include bradykinesia, muscular rigidity, and resting tremor.

Although PD is currently considered a multifactorial disorder, in which both environmental factors and genetic susceptibilities contribute to its pathogenesis (Cannon and Greenamyre 2013), the cellular and molecular processes underlying the preferential dopaminergic degeneration are not fully elucidated. In this scenario, the presence of dopamine (DA) itself has been proposed as one of the pathogenic factors (Bisaglia et al. 2014; Bisaglia et al. 2013). At cellular level, after the synthesis in the cytosol, DA is stored inside synaptic vesicles, where the low pH value can stabilize the neurotransmitter. Cytosolic DA can otherwise be catabolized at the level of outer mitochondrial membrane by the action of monoamine oxidase (MAO) through a reaction that leads to the formation of hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL). The accumulation of DA into the cytosol, as consequences of impaired synthesis, accumulation into the synaptic vesicles, metabolism, or reuptake from the synaptic cleft, can lead to spontaneous oxidation, due to the neutral pH, and generate both reactive oxygen species (ROS) and dopamine-quinones (DAQs), highly reactive electrophilic molecules (Graham 1978; Klegeris et al. 1995). In vivo, this last pathway is manifested by the presence in the dopaminergic neurons of neuromelanin, a dark pigment mainly formed by DAQs (Zucca et al. 2014). Both ROS and DAQs might have cytotoxic effects and impair the neuronal survival. In fact, ROS can damage cellular components such as lipids, proteins, and DNA, whereas the electron-deficient quinones can react with and covalently modify cellular nucleophiles such as cysteine

✉ Marco Bisaglia
marco.bisaglia@unipd.it

[1] Molecular Physiology and Biophysics Unit, Department of Biology, University of Padova, Padova, Italy

[2] Present address: Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

residues (Bisaglia et al. 2014). Therefore, the presence of cytosolic DA could be responsible for increased levels of oxidative damage, a pathological condition associated with the onset and/or progression of PD (Henchcliffe and Beal 2008). To cope with the physiological production of ROS, cells express numerous antioxidant enzymes. Among them, superoxide dismutases (SODs) are responsible for the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide, therefore playing a pivotal role in the antioxidant response. In fact, superoxide radicals are often considered the "primary" ROS as they can further react with other molecules to produce more reactive "secondary" ROS, such as hydroxyl radicals and peroxynitrites (De Lazzari et al. 2018; Willems et al. 2015). Therefore, SODs are often regarded as the first line of defense against ROS (De Lazzari et al. 2018). Two different SOD isoenzymes are present inside cells, referred to as SOD1 and SOD2 (Zelko et al. 2002). SOD1 is a copper/zinc protein mainly located in the cytosol, but it is also present in the mitochondrial intermembrane space, in peroxisomes, and in the nucleus. SOD2 is a mitochondrial manganese protein, responsible for the elimination of superoxide anions produced during the oxidative phosphorylation.

Considering the involvement of oxidative stress in the onset and/or progression of PD, it is not surprising that antioxidant molecules have been largely studied for their potential therapeutic properties, even though the results reached until now have been somehow disappointing (Filograna et al. 2016a). A main issue with antioxidants is that most of them do not target the primary cause of oxidative stress, i.e., excessive superoxide anion production, but rather the downstream products, such as hydrogen peroxides, hydroxyl radicals, or peroxynitrites (De Lazzari et al. 2018). We thus decided to explore whether the use of a more specific strategy targeted against the origin of oxidative damage could be more effective. In this frame, we have recently demonstrated the protective role of SOD1 and SOD2 in both sporadic and genetic models of PD (Biosa et al. 2018b; Filograna et al. 2016b). Following up on these promising results, in the present work, we performed a systematic flow cytometry study with the aim of verifying whether the overexpression of either SOD1 or SOD2 might rescue the toxic effects induced by the cytosolic accumulation of DA in human neuroblastoma SH-SY5Y cell line.

Materials and Methods

Cell Culture Human neuroblastoma SH-SY5Y cells (IST, Genova, Italy) were cultured in a 1:1 mixture of Ham's F12 (F12) and Dulbecco Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% (*v*/*v*) fetal bovine serum, in a 5% CO_2 humidified incubator at 37 °C. The cell medium was replaced every 3 days, and the cells were subcultured once confluence was reached. In every experiment, cells were used at early passages (P1–5 after purchase). SOD1 and SOD2 constitutively overexpressing SH-SY5Y cells were produced as previously described (Filograna et al. 2016b). For cell differentiation, 1.5×10^5cells/well were plated in 12-well plates and differentiation was induced by adding 10 μM of retinoic acid (RA). Fresh medium containing RA was replaced every 2 days.

Flow Cytometry Wild-type and SOD-overexpressing cells were transferred on 6-well plates (2.5×10^5 cells/well) in the presence of DMEM-F12 medium without phenol red, supplemented with 10% (*v*/*v*) fetal bovine serum. The absence of phenol red is mandatory for the following analysis because, otherwise, it can interfere with the propidium iodide (PI) detection. The next day, cells were treated with DA or DAQs. In brief, to assess DA-corresponding toxicity, different amounts of DA were directly added to the culture medium, while, to evaluate DAQ-related toxicity, cells were first treated with different concentrations of DA in the presence of 0.025 U/μl of tyrosinase (or with tyrosinase alone as control) in Hanks' balanced salt solution (HBSS) without bicarbonate for 1 h at 37 °C and, then, culture medium was replaced with complete medium without phenol red.

After 24 h of treatment, culture media containing cell debris were transferred in 15-ml high-clarity polypropylene conical tubes (BD Falcon). Adherent cells were washed with phosphate buffer saline, detached by using 150 μl of 5 U/ml of papain protease (Worthington), and centrifuged 5 min at 140 × *g* in the presence of the corresponding culture medium. After two washes with 3 ml of FACS buffer (phosphate buffer saline with 1% bovine serum albumin), cells were resuspended in 190 μl of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, and 1 g/l glucose). Cell suspensions were then transferred into 5-ml round-bottom tubes, and 10 μl of propidium iodide (20 μg/ml) was added and incubated for a few seconds at room temperature. Samples were analyzed on Canto II flow cytometer (BD Bioscience), and 5000 gated events were acquired.

Western Blot Analysis After 6 days of differentiation in the presence of RA, cells were collected and lysed on ice in 50 μl of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate supplemented with protease cocktail inhibitor (P8340, Sigma). Cell lysates were stored at − 80 °C until loading in SDS-PAGE. Protein concentration was determined by BCA assay (Pierce), and equal amounts of total proteins (25 μg) were separated in a 10% poly-acrylamideTris-Glycine-SDS gel and blotted on a PVDF membrane (Bio-Rad) in semi-dry conditions using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). Immunoblotting was performed

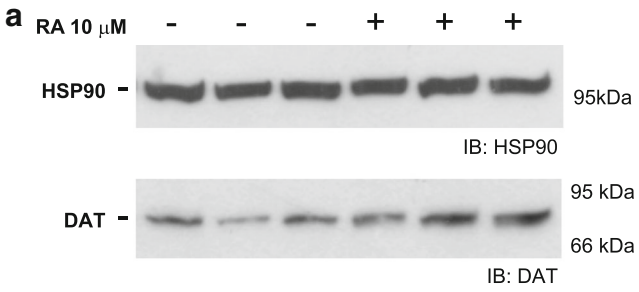
by incubation with the primary antibodies (anti-DAT 1:1000, Sigma-Aldrich; anti-HSP90 1:10000, OriGene) overnight at 4 °C, followed by incubation with secondary antibodies conjugated with HorseRadish-Peroxidase (anti-rabbit IgG-HRP 1:15000, Sigma-Aldrich; anti-mouse IgG-HRP 1:80000, Sigma-Aldrich) for 1 h at room temperature. Immunoreactive proteins were visualized using enhanced chemio-luminescence (ECL, GE Healthcare). Densitometry was carried out using Image J Software.

Results

The Toxicity of Cytosolic DA Is Mostly Caused by ROS in Human SH-SY5Y Neuroblastoma Cells In order to explore the toxicity caused by the cytosolic accumulation of DA, we first analyzed the cellular damage induced by different concentrations of DA added into the cellular medium. To this aim, human SH-SY5Y neuroblastoma cells were used. This cell line is frequently chosen in PD research because of its human origin and catecholaminergic neuronal properties (Filograna et al. 2015). Here we have verified that these cells express the presynaptic DA transporter DAT, which is responsible, in physiological conditions, for the reuptake of the DA from the synaptic cleft and, in our experimental conditions, for the intracellular accumulation of DA once added into the culture medium. More specifically, we evaluated the protein levels in both undifferentiated cells and upon differentiation in the presence of 10 μM retinoic acid (RA). As represented in Fig. 1, DAT levels were not statistically different between the two conditions tested. For this reason, in the following experiments, we worked with undifferentiated cells.

Cellular toxicity was measured by flow cytometry, using propidium iodide (PI) to measure cell death. PI is a membrane-impermeant fluorescent dye, which does not usually accumulate inside viable cells whereas it is able to penetrate through the damaged, permeable membrane of dying cells.

As represented in Fig. 2, the addition of DA into the cellular medium increases the number of PI-positive cells in a dose-dependent manner. These results are in agreement with previously published data obtained in SH-SY5Y and other cell lines (Banerjee et al. 2014; Bisaglia et al. 2010a; Emdadul Haque et al. 2003; Ganguly et al. 2019; Gimenez-Xavier et al. 2006; Greggio et al. 2005; Izumi et al. 2005; Jana et al. 2011; Lai and Yu 1997). As the auto-oxidation of cytosolic DA produces DAQs and ROS, which are both highly reactive, we proceeded to analyze and discriminate the contribution of each of these reactive species in terms of cellular toxicity. Therefore, we investigated the effect induced by DAQs that were generated from DA by the action of the enzyme tyrosinase, which does not produce radical species in solution (Solomon et al. 1996). When cells were treated with DAQs,



b

SH-SY5Y	DAT/HSP90
Undifferentiated	0.41 ± 0.08
RA 10 μM	0.75 ± 0.13

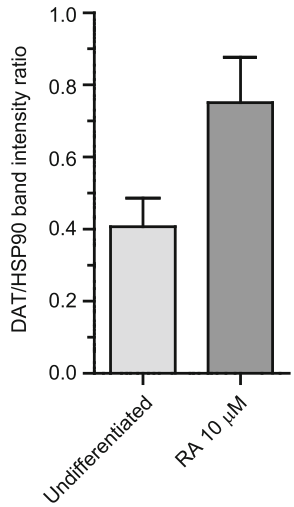
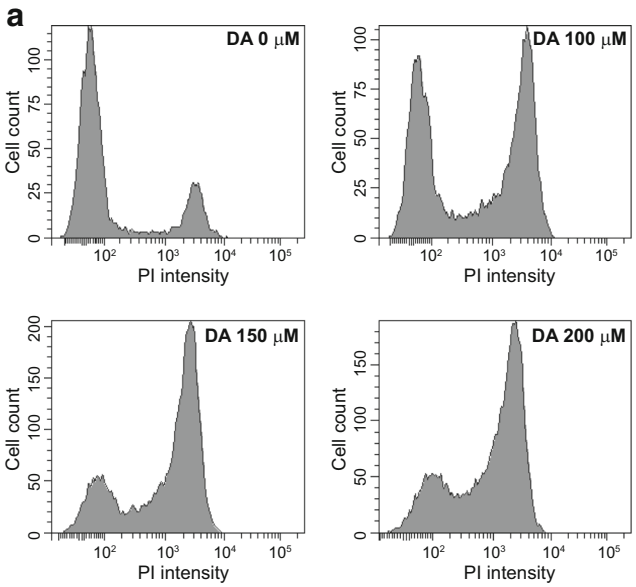


Fig. 1 **a** Western blot analysis and **b** densitometric quantification of DAT in undifferentiated and RA-differentiated SH-SY5Y cells. The quantification was obtained using Image J software. The HSP90 signal was used as loading control. Data are expressed as mean ± SEM of three independent experiments. Statistical significance was determined by Mann-Whitney non-parametric test, and no difference was observed

the percentage of PI-positive cells increased in comparison with cells treated with tyrosinase alone in the absence of DA (Fig. 3), passing from ~ 13 to ~ 25%. Nevertheless, the toxicity induced by DAQs did not show a clear dose-response relationship and moreover, it was much less pronounced than that observed when treating cells with DA (Figs. 2 and 3). These results suggest that, in SH-SY5Y cell cultures, the cytotoxic effects caused by DA auto-oxidation appear more related to the formation of ROS rather than to the production of DAQs.

SOD1 and SOD2 Overexpression Protects SH-SY5Y Cells from DA-Related Toxicity Considering that oxidative stress has been recognized as an important factor involved in the onset and/or progression of PD, we then tested the protective potential of



b

SH-SY5Y WT	DA 0 μM	DA 100 μM	DA 150 μM	DA 200 μM
PI -	85.7 ± 1.4	54 ± 3	31 ± 2	26.1 ± 1.9
PI +	14.3 ± 1.4	46 ± 3	69 ± 2	73.9 ± 1.9

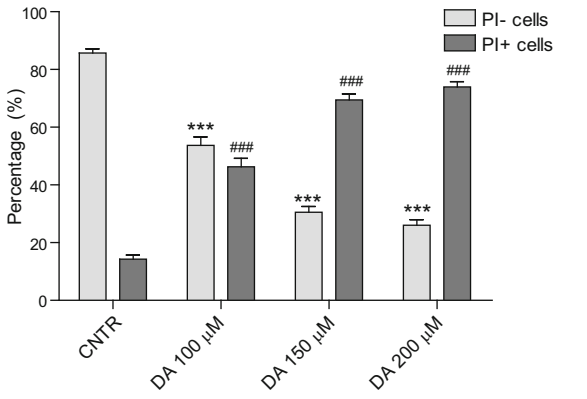
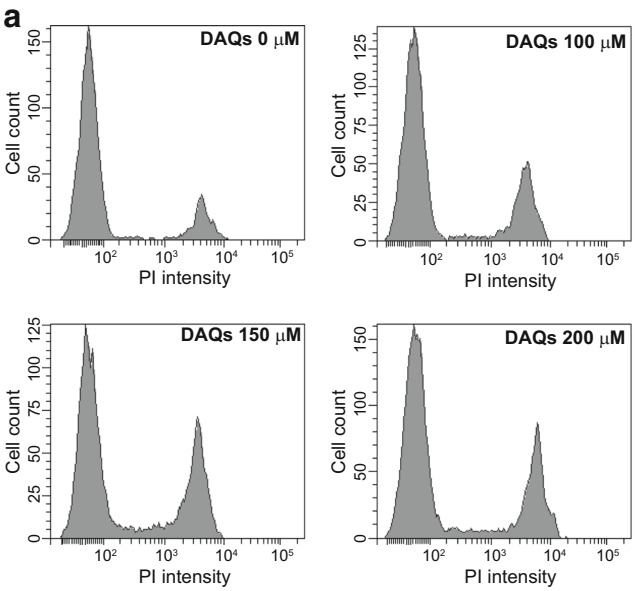


Fig. 2 **a** Representative pictures of the cytofluorimetric analysis carried out on wild-type SH-SY5Y cells. In each graph, the correlation between the number of cells and the PI fluorescence intensity is represented at a given concentration of DA. **b** Table and histogram indicating the percentage of PI-negative and PI-positive cells at different concentrations of DA. Data are expressed as mean ± SEM of at least 3 independent experiments. Statistical significance was assessed by one-way ANOVA with Bonferroni correction (*** or ### $p < 0.001$). For the sake of clarity, only statistical significance relative to DA-untreated cells is reported (* refers to PI-negative cells; # refers to PI-positive cells)

SODs against the cellular toxicity related to the intracellular accumulation of DA. The rationale behind our choice comes from the fact that both DA metabolism and DA auto-oxidation produce superoxide radicals and that these radical species are often the first step of the pathway that eventually leads to the cellular oxidative damage. SOD1 or SOD2 constitutively overexpressing cells were previously characterized in our lab (Filograna et al. 2016b). The overexpression levels of SOD1 and SOD2 are, respectively, 4.6 ± 1.3 and 5.7 ± 1.3 (Filograna et al. 2016b).

When SOD1-overexpressing cells were incubated with increasing amounts of DA, we could still detect an increased



b

SH-SY5Y WT	DAQs 0 μM	DAQs 100 μM	DAQs 150 μM	DAQs 200 μM
PI -	86.8 ± 1.3	73 ± 4	75 ± 2	75 ± 3
PI +	13.2 ± 1.3	27 ± 4	25 ± 2	25 ± 3

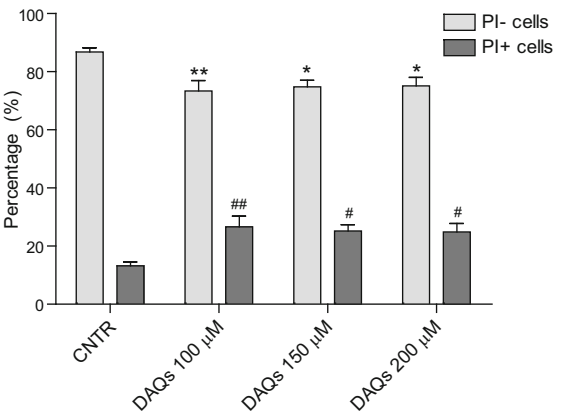


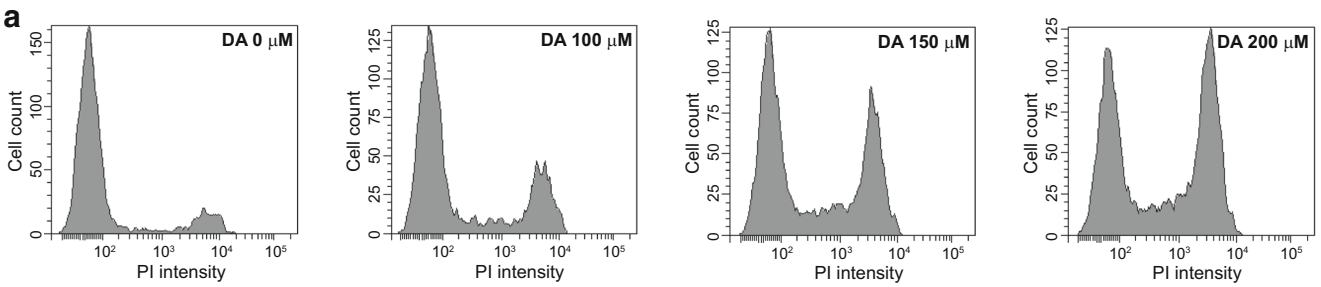
Fig. 3 **a** Representative pictures of the cytofluorimetric analysis carried out on wild-type SH-SY5Y cells. In each graph, the correlation between the number of cells and the PI fluorescence intensity is represented at a given concentration of DAQs. **b** Table and histogram indicating the percentage of PI-negative and PI-positive cells at different concentrations of DAQs. Data are expressed as mean ± SEM of at least 3 independent experiments. Statistical significance was assessed by one-way ANOVA with Bonferroni correction (* or # $p < 0.05$, ** or ## $p < 0.01$). For the sake of clarity, only statistical significance relative to DAQ-untreated cells is reported (* refers to PI-negative cells; # refers to PI-positive cells)

number of PI-positive cells in comparison with the control (Fig. 4a), although the cytotoxicity was significantly reduced in comparison with the one observed in wild-type cells (Fig. 4b). This result can be emphasized by normalizing each series of data with respect to the corresponding control (untreated cells). As shown in Fig. 4c, the percentage of viable cells increases from 63 ± 4%, 35.5 ± 1.8%, and 30 ± 2% to 88 ± 4%, 80 ± 7%, and 47 ± 4% in the presence of 100 μM, 150 μM and 200 μM DA, respectively.

A similar behavior was observed with SOD2-overexpressing cells. As represented in Fig. 5a and b, the addition of 100 μM or 200 μM DA in the cellular medium of SOD2-overexpressing cultures increased the percentage of PI-positive cells from 22.6 ± 1.2% to 32 ± 2% and 66 ± 2%, respectively. However, as in the case of SOD1, these values were remarkably lower in comparison with those observed in wild-type cells treated with the same concentration of neurotransmitter. The normalization of each series of data to the corresponding controls (Fig. 5c) confirmed that the SOD2-mediated protection was statistically significant. Taken together, these data showed that the antioxidant enzyme activity of SOD1 and SOD2 plays a beneficial role against the DA-derived toxicity.

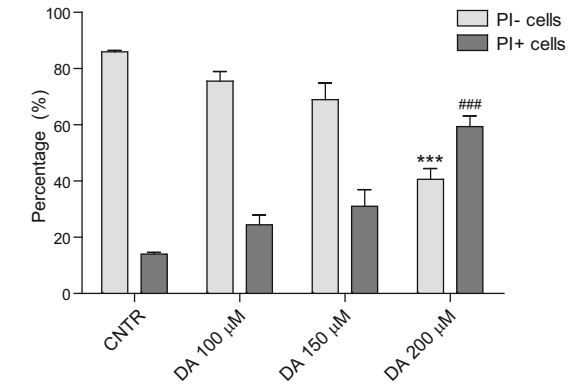
SOD1 and SOD2 Do Not Protect SH-SY5Y Cells from DAQ Toxicity Once demonstrated the protective role of SODs against the oxidative damage induced by the presence of DA, we evaluated whether these two enzymes were also able to protect cells from DAQ toxicity. As previously described, to avoid the presence of ROS, DAQs were generated through the use of the enzyme tyrosinase.

As shown in Fig. 6a and b, the treatment of SOD1-overexpressing cells with 100 μM DA plus tyrosinase determined the increase of PI fluorescent signal in comparison with cells treated only with tyrosinase. Specifically, the percentage of PI-positive cells raised from 9.2 ± 1.4 to 19 ± 2. When we



b

SH-SY5Y SOD1	DA 0 μM	DA 100 μM	DA 150 μM	DA 200 μM
PI -	85.9 ± 0.6	76 ± 3	69 ± 6	41 ± 4
PI +	14.1 ± 0.6	24 ± 3	31 ± 6	59 ± 4



c

WT vs SOD1	DA 100 μM	DA 150 μM	DA 200 μM
PI - (WT)	63 ± 4	35.5 ± 1.8	30 ± 2
PI - (SOD1)	88 ± 4	80 ± 7	47 ± 4

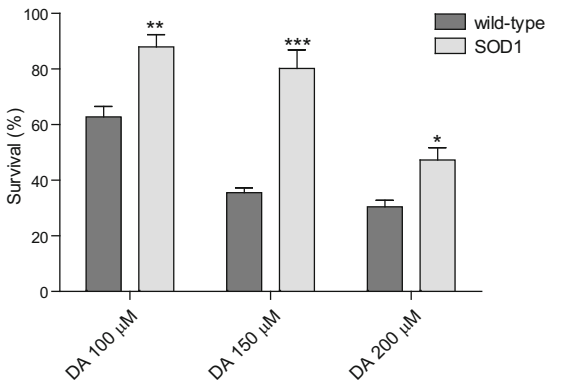
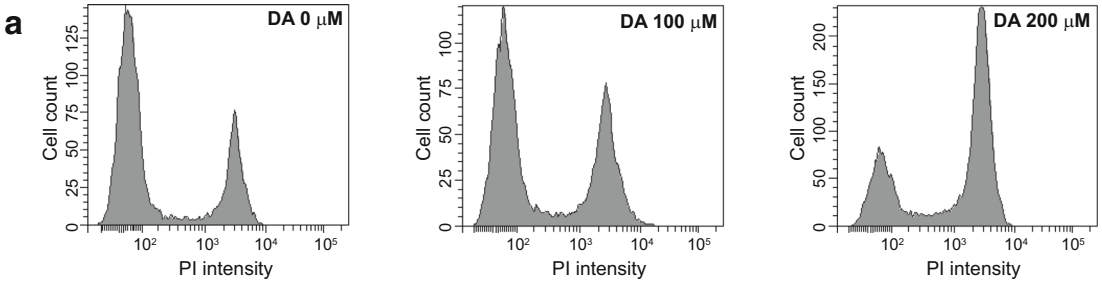
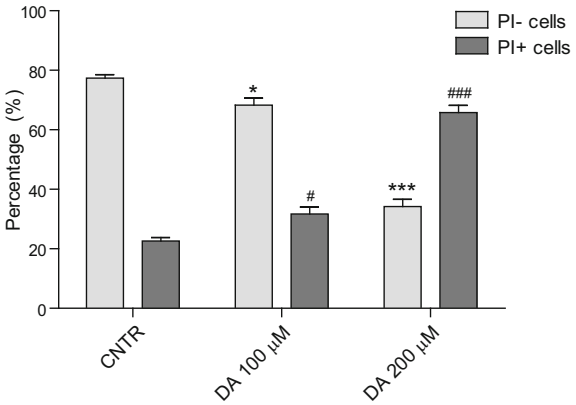


Fig. 4 **a** Representative pictures of the cytofluorimetric analysis carried out on SOD1-overexpressing SH-SY5Y cells. In each graph, the correlation between the number of cells and the PI fluorescence intensity is represented at a given concentration of DA. **b** Table and histogram indicating the percentage of PI-negative and PI-positive cells at different concentrations of DA. Data are expressed as mean ± SEM of at least 3 independent experiments. Statistical significance was assessed by one-way ANOVA with Bonferroni correction (***$p < 0.001$). For the sake of clarity, only statistical significance relative to DA-untreated cells is reported (* refers to PI-negative cells; # refers to PI-positive cells). **c** Table and histogram indicating the percentage of PI-negative at different concentration of dopamine, normalized to the corresponding control (untreated cells). Statistical significance was determined by *t* test, comparing SOD1-overexpressing cells with wild-type cells at each DA concentration tested (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)



b

SH-SY5Y SOD2	DA 0 μM	DA 100 μM	DA 200 μM
PI -	77.4 ± 1.2	68 ± 2	34 ± 2
PI +	22.6 ± 1.2	32 ± 2	66 ± 2



c

WT vs SOD2	DA 100 μM	DA 200 μM
PI - (WT)	63 ± 4	30 ± 2
PI - (SOD2)	88 ± 2	44 ± 4

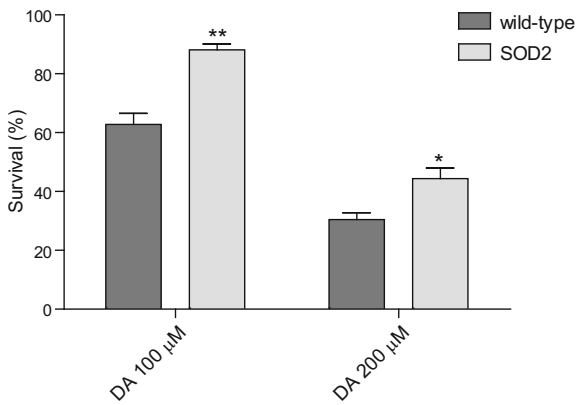


Fig. 5 **a** Representative pictures of the cytofluorimetric analysis carried out on SOD2-overexpressing SH-SY5Y cells. In each graph, the correlation between the number of cells and the PI fluorescence intensity is represented at a given concentration of DA. **b** Table and histogram indicating the percentage of PI-negative and PI-positive cells at different concentrations of DA. Data are expressed as mean ± SEM of at least 3 independent experiments. Statistical significance was assessed by one-way ANOVA with Bonferroni correction (* or $^{\#}$ $p < 0.05$, *** or $^{\#\#\#}$ $p < 0.001$). For the sake of clarity, only statistical significance relative to DA-untreated cells is reported (* refers to PI-negative cells; $^{\#}$ refers to PI-positive cells). **c** Table and histogram indicating the percentage of PI-negative cells at different concentration of dopamine, normalized to the corresponding control (untreated cells). Statistical significance was determined by *t* test, comparing SOD2-overexpressing cells with wild-type cells at each DA concentration tested (*$p < 0.05$, **$p < 0.01$)

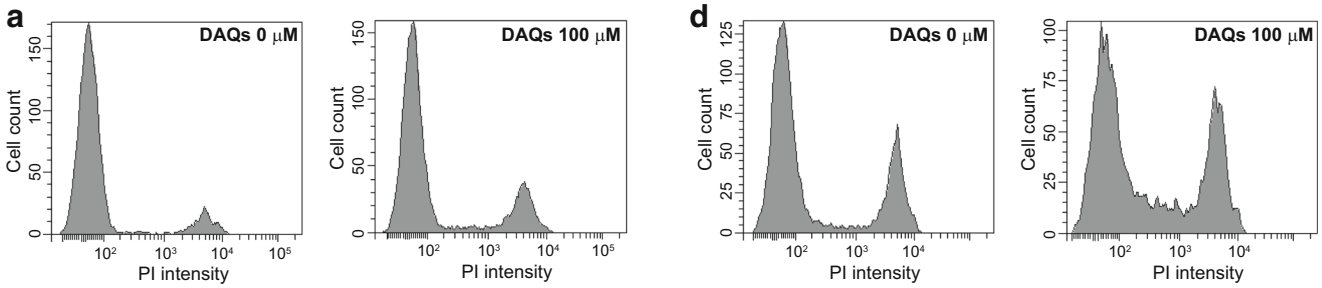
normalized the fraction of viable cells upon treatment with DAQs to the viability observed in the presence of tyrosinase alone and compared the value to that obtained in wild-type cells, we observed a small increment in the percentage of PI-negative cells, which however was not statistically significant (Fig. 6c).

When we then tested the protective role of SOD2 against DAQs, the effects were very similar to that exerted by SOD1. When SOD2-overexpressing cells were treated with 100 μM DA plus tyrosinase, we observed a slight increase of PI-positive cells in comparison with cells treated with tyrosinase alone (Fig. 6d, e). After the normalization with respect to the corresponding control, the comparison of the data obtained with wild-type or SOD2-overexpressing cells showed a 10% reduction of PI-positive cells, even though this difference was again not statistically significant (Fig. 6f).

In summary, our data seem to suggest that the protection of SOD1 and SOD2 against DA toxicity depends mainly on their ability to eliminate ROS produced during the metabolism of DA, while the enzymes do not seem to ameliorate the effects induced by the toxicity of DAQs.

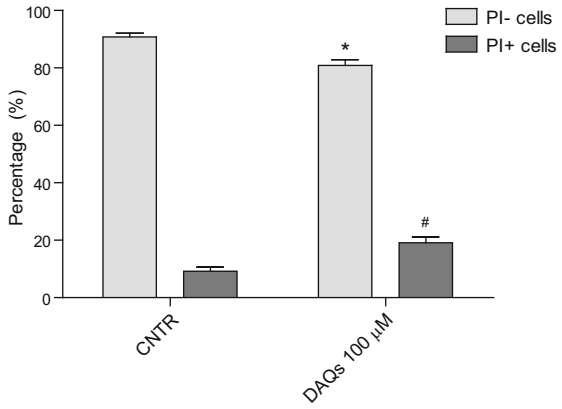
Discussion

In this work, we have characterized the toxicity of DA in human neuroblastoma SH-SY5Y cells, discriminating the effects derived from the production of ROS from those related to the presence of DAQs. To this aim, DAQs were produced using the enzyme tyrosinase, which does not produce radical species in solution. Indeed, tyrosinase uses molecular oxygen as co-substrate to catalyze the hydroxylation of monophenols to diphenols (monophenolase activity) and the oxidation of diphenols to quinones (diphenolase activity). Both reactions take place without the production of ROS (Solomon et al. 1996), so that, using this strategy, we were able to focus



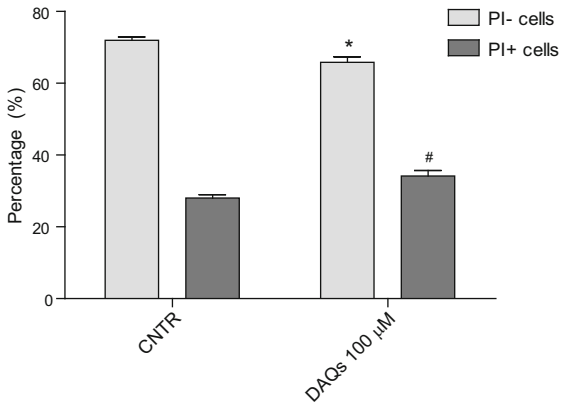
b

SH-SY5Y SOD1	DAQs 0 μM	DAQs 100 μM
PI -	90.8 ± 1.4	81 ± 2
PI +	9.2 ± 1.4	19 ± 2



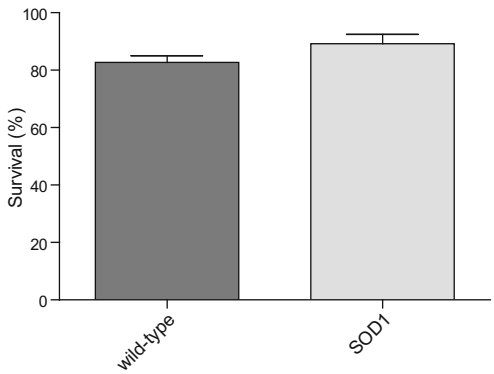
e

SH-SY5Y SOD2	DAQs 0 μM	DAQs 100 μM
PI -	72 ± 1.0	65.8 ± 1.5
PI +	28 ± 1.0	34.2 ± 1.5



c

WT vs SOD1	DAQs 100 μM
PI - (WT)	83 ± 2
PI - (SOD1)	89 ± 3



f

WT vs SOD2	DAQs 100 μM
PI - (WT)	83 ± 2
PI - (SOD1)	92 ± 3

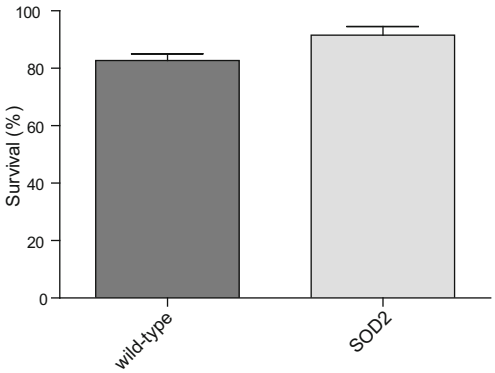


Fig. 6 **a**, **d** Representative pictures of the cytofluorimetric analysis carried out on (**a**) SOD1- or (**d**) SOD2-overexpressing SH-SY5Y cells. In each graph, the correlation between the number of cells and the PI fluorescence intensity is represented at a given concentration of DAQs. **b**, **e** Table and histogram indicating the percentage of PI-negative and PI-positive cells at different concentrations of DAQs in (**b**) SOD1- or (**e**) SOD2-overexpressing SH-SY5Y cells. Data are expressed as mean ± SEM of at least 3 independent experiments. Statistical significance was determined by *t* test, by comparing cells treated with 100 μM DAQs and the untreated controls (* or $^{\#}$ $p < 0.05$, where * refers to PI-negative cells and $^{\#}$ refers to PI-positive cells). **c**, **f** Table and histogram indicating the percentage of PI-negative in the presence of 100 μM DAQs, normalized to the corresponding control (untreated cells). Statistical significance was determined by *t* test, comparing SOD-overexpressing cells with wild-type cells

exclusively on the untoward effects of DAQs. DA-related toxicity was assessed by adding the neurotransmitter directly into the culture medium, because SH-SY5Y cells express the DA transporter DAT, which mediates the intracellular uptake of DA (Gimenez-Xavier et al. 2006). Also DAQs were generated in the culture medium and we have previously demonstrated their cellular uptake (Biosa et al. 2018a). Nevertheless, the way through which DAQs enter cells is still an open question. Non-specific organic cation transporters (OCT) could be involved, even though we do not have any experimental evidence. The harmful effects measured by flow cytometry indicate that, in our experimental conditions, DA is much more detrimental to cell integrity in comparison with DAQs, with a toxicity that is approximately 3 times higher at the highest concentration used. This suggests that the cellular damage arising from DA-derived ROS production is quantitatively more important than the toxic effects mediated by DAQs. Our results suggest that the presence of DA could make dopaminergic neurons more sensitive than other neuronal populations to alterations in the cellular oxidative state. As oxidative conditions are often associated to aging, our results could rationalize the fact that aging represents the most important risk factor for PD. It is also worth mentioning that, although not quantitatively predominant, DAQs represent very reactive molecules exclusively formed in dopaminergic neurons. As a consequence, DAQ toxicity could accumulate over long periods of time, contributing to the preferential degeneration of this class of neurons.

After having evaluated the DA-related toxicity, through the use of cell lines constitutively overexpressing the antioxidant enzymes SOD1 or SOD2, we assessed their potential protective role in our experimental paradigms. Previous works already demonstrated that exogenously added SOD1 in the culture medium was able to protect SH-SY5Y cells from DA-induced toxicity also suggesting a possible role of the protein in inhibiting the generation of DA-semiquinone radicals (Emdadul Haque et al. 2003; Izumi et al. 2005). In our work, we evaluated the potential protection exerted by endogenous SOD overexpression. Moreover, as SOD1 and SOD2 have two distinct cellular compartmentalizations, being SOD2 found in the mitochondrial matrix while SOD1 is mainly a cytosolic protein, in this way, it has been possible to discriminate whether the DA-related toxicity only depends on the mitochondrial or cytosolic production of ROS or if both mitochondria and cytosolic processes contribute in promoting cell death. Our data emphasize the beneficial effects of both SOD1 and SOD2. More specifically, considering the similar protective effects observed with either SOD1 or SOD2, we can infer that the damage associated to DA depends on both cytosolic and mitochondrial production of ROS. This can be rationalized, on one hand, considering that both DA autoxidation and its degradation by MAO are cytosolic events leading to the formation of free radical species, including superoxide radicals, and, on the other hand, by the fact that oxidative stress and mitochondrial dysfunction are strictly associated (Henchcliffe and Beal 2008). In contrast, we did not observe significant protection exerted by SODs against the harmful effects associated to the presence of DAQs. These results can be in part explained considering that DAQs were formed using tyrosinase without the concomitant production of ROS, but the analysis is probably more complex. In fact, previously published works from our and other groups have demonstrated the capability of DAQs to induce the opening of the mitochondrial transition pore (Berman and Hastings 1999; Biosa et al. 2018a; Bisaglia et al. 2010b) at the level of the inner mitochondrial membrane. The consequence is a rapid loss of mitochondrial membrane potential and ATP levels, which cause a loss of ion homeostasis and cell integrity, which eventually results in necrosis (Bonora and Pinton 2014). Moreover, the opening of the pore and the release of the proapoptotic factors, such as cytochrome C, are considered additional pathways responsible for cell death, supporting a role for the mitochondrial permeability transition also in apoptotic cell death (Bonora and Pinton 2014). Even though it is known that mitochondrial permeability transition increases the levels of ROS (Zorov et al. 2014), the data presented here suggest that other ROS-independent pathways play, most probably, a major role in DAQ-mediated cell damaging.

The results reported in this study might have a potential therapeutic relevance for the cure of Parkinson's disease. Albeit PD is a multifactorial disorder and its pathogenesis can result from the interaction of a variety of cellular processes, oxidative injury is recognized as a key factor involved in PD progression. In fact, alterations in redox homeostasis can trigger many dysfunctional processes associated to the disease, such as mitochondria dysfunction, endoplasmic reticulum (ER) stress, impaired autophagy, and protein aggregation (Puspita et al. 2017; Zeng et al. 2018). As aforementioned, a very strong correlation exists between oxidative stress and mitochondria dysfunction. If on one hand mitochondria represent the main source of superoxide radicals inside cells, on the other hand, they are particularly susceptible to oxidative damage, especially at the mitochondrial DNA level (Bender et al. 2006). Moreover, an excessive production of ROS can alter the intracellular calcium signaling, promoting calcium influx into mitochondria at specialized junctions with ER (Ermak and Davies 2002; Hayashi et al. 2009), which further increases mitochondrial ROS production and ER stress in a vicious cycle of calcium release and ROS production (Malhotra and Kaufman 2007). Proteostatic dysfunction, which is another key feature of PD, appears to be exacerbated by oxidative injury, too. In fact, ROS have been demonstrated to induce α-synuclein aggregation and to inhibit lysosomes through oxidative damage to lysosomal proteins like glucocerebrosidase, as recently reviewed (Surmeier 2018). It is worth mentioning that increased levels of calcium can

directly promote α-synuclein aggregation and mitochondrial damage should increase mitophagy, lowering the autophagic capacity of neuron and their ability to cope with intracellular aggregates like α-synuclein fibrils (Surmeier 2018), creating a connection among different pathological processes centered around oxidative injury. Interestingly, in dopaminergic neurons derived from induced pluripotent stem cells from PD patients, it has been demonstrated that mitochondrial dysfunction triggers a dopamine-dependent toxic cascade which results in α-synuclein aggregation and lysosomal dysfunction (Burbulla et al. 2017). If the presence of DA makes dopaminergic neurons more sensitive than other neuronal populations to alterations in the cellular oxidative state, as confirmed by our results, and if superoxide radicals play a major role in PD-related oxidative injury, their selective removal could be protective, as our data seem to suggest. Accordingly, we have previously demonstrated the beneficial effects of overexpressing SOD1 or SOD2 in paraquat-based cellular and animal models of PD (Filograna et al. 2016b). Moreover, SOD1 or SOD2 overexpression was able to rescue, both in vitro and in vivo, the pathological phenotypes in two genetic models of PD, based, respectively, on PINK1 or Parkin loss (Biosa et al. 2018b). Even though SODs cannot be exploited, by themselves, for therapeutic purposes in the context of PD, this work further supports the evaluation of the therapeutic potential of small synthetic antioxidant molecules specifically designed to cope with superoxide radicals.

References

Banerjee K, Munshi S, Sen O, Pramanik V, Roy Mukherjee T, Chakrabarti S (2014) Dopamine cytotoxicity involves both oxidative and nonoxidative pathways in SH-SY5Y cells: potential role of alpha-synuclein overexpression and proteasomal inhibition in the etiopathogenesis of Parkinson's disease. Parkinsons Dis 2014: 878935. https://doi.org/10.1155/2014/878935

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet 38:515–517. https://doi.org/10.1038/ng1769

Berman SB, Hastings TG (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J Neurochem 73: 1127–1137

Biosa A, Arduini I, Soriano ME, Giorgio V, Bernardi P, Bisaglia M, Bubacco L (2018a) Dopamine oxidation products as mitochondrial endotoxins, a potential molecular mechanism for preferential neurodegeneration in Parkinson's disease. ACS Chem Neurosci 9:2849–2858. https://doi.org/10.1021/acschemneuro.8b00276

Biosa A, Sanchez-Martinez A, Filograna R, Terriente-Felix A, Alam SM, Beltramini M, Bubacco L, Bisaglia M, Whitworth AJ (2018b) Superoxide dismutating molecules rescue the toxic effects of PINK1 and parkin loss. Hum Mol Genet 27:1618–1629. https://doi.org/10.1093/hmg/ddy069

Bisaglia M, Greggio E, Maric D, Miller DW, Cookson MR, Bubacco L (2010a)Alpha-synuclein overexpression increases dopamine toxicity in BE2-M17 cells. BMC Neurosci 11:41. https://doi.org/10.1186/1471-2202-11-41

Bisaglia M, Soriano ME, Arduini I, Mammi S, Bubacco L (2010b) Molecular characterization of dopamine-derived quinones reactivity toward NADH and glutathione: implications for mitochondrial dysfunction in Parkinson disease. Biochim Biophys Acta 1802:699–706. https://doi.org/10.1016/j.bbadis.2010.06.006

Bisaglia M, Greggio E, Beltramini M, Bubacco L (2013) Dysfunction of dopamine homeostasis: clues in the hunt for novel Parkinson's disease therapies. FASEB J 27:2101–2110. https://doi.org/10.1096/fj.12-226852

Bisaglia M, Filograna R, Beltramini M, Bubacco L (2014) Are dopamine derivatives implicated in the pathogenesis of Parkinson's disease? Ageing Res Rev 13:107–114. https://doi.org/10.1016/j.arr.2013.12.009

Bonora M, Pinton P (2014) The mitochondrial permeability transition pore and cancer: molecular mechanisms involved in cell death. Front Oncol 4:302. https://doi.org/10.3389/fonc.2014.00302

Burbulla LF, Song P, Mazzulli JR, Zampese E, Wong YC, Jeon S, Santos DP, Blanz J, Obermaier CD, Strojny C, Savas JN, Kiskinis E, Zhuang X, Krüger R, Surmeier DJ, Krainc D (2017) Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in Parkinson's disease. Science 357:1255–1261. https://doi.org/10.1126/science.aam9080

Cannon JR, Greenamyre JT (2013)Gene-environment interactions in Parkinson's disease: specific evidence in humans and mammalian models. Neurobiol Dis 57:38–46. https://doi.org/10.1016/j.nbd.2012.06.025

De Lazzari F, Bubacco L, Whitworth AJ, Bisaglia M (2018) Superoxide radical dismutation as new therapeutic strategy in Parkinson's disease. Aging Dis 9:716–728. https://doi.org/10.14336/AD.2017.1018

Emdadul Haque M, Asanuma M, Higashi Y, Miyazaki I, Tanaka K, Ogawa N (2003)Apoptosis-inducing neurotoxicity of dopamine and its metabolites via reactive quinone generation in neuroblastoma cells. Biochim Biophys Acta 1619:39–52

Ermak G, Davies KJ (2002) Calcium and oxidative stress: from cell signaling to cell death. Mol Immunol 38:713–721

Filograna R, Civiero L, Ferrari V, Codolo G, Greggio E, Bubacco L, Beltramini M, Bisaglia M (2015) Analysis of the catecholaminergic phenotype in human SH-SY5Y and BE(2)-M17 neuroblastoma cell lines upon differentiation. PLoS One 10:e0136769. https://doi.org/10.1371/journal.pone.0136769

Filograna R, Beltramini M, Bubacco L, Bisaglia M (2016a)Anti-oxidants in Parkinson's disease therapy: a critical point of. View Curr Neuropharmacol 14:260–271. https://doi.org/10.2174/1570159X1403160303151355

Filograna R, Godena VK, Sanchez-Martinez A, Ferrari E, Casella L, Beltramini M, Bubacco L, Whitworth AJ, Bisaglia M (2016b) Superoxide dismutase (SOD)-mimetic M40403 is protective in cell and fly models of paraquat toxicity: implications for Parkinson disease. J Biol Chem 291:9257–9267. https://doi.org/10.1074/jbc.M115.708057

Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55:259–272

Ganguly U, Ganguly A, Sen O, Ganguly G, Cappai R, Sahoo A, Chakrabarti S (2019) Dopamine cytotoxicity on SH-SY5Y cells: involvement of alpha-synuclein and relevance in the neurodegeneration of sporadic Parkinson's disease. Neurotox Res 35:898–907. https://doi.org/10.1007/s12640-019-0001-0

Gimenez-Xavier P et al (2006) The decrease of NAD(P) H has a prominent role in dopamine toxicity. Biochim Biophys Acta 1762:564–574. https://doi.org/10.1016/j.bbadis.2006.02.003

Graham DG (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 14:633–643

Greggio E, Bergantino E, Carter D, Ahmad R, Costin GE, Hearing VJ, Clarimon J, Singleton A, Eerola J, Hellstrom O, Tienari PJ, Miller DW, Beilina A, Bubacco L, Cookson MR (2005) Tyrosinase exacerbates dopamine toxicity but is not genetically associated with Parkinson's disease. J Neurochem 93:246–256. https://doi.org/10.1111/j.1471-4159.2005.03019.x

Hayashi T, Rizzuto R, Hajnoczky G, Su TP (2009) MAM: more than just a housekeeper. Trends Cell Biol 19:81–88. https://doi.org/10.1016/j.tcb.2008.12.002

Henchcliffe C, Beal MF (2008) Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. Nat Clin Pract Neurol 4:600–609. https://doi.org/10.1038/ncpneuro0924

Izumi Y, Sawada H, Yamamoto N, Kume T, Katsuki H, Shimohama S, Akaike A (2005) Iron accelerates the conversion of dopamine-oxidized intermediates into melanin and provides protection in SH-SY5Y cells. J Neurosci Res 82:126–137. https://doi.org/10.1002/jnr.20595

Jana S, Sinha M, Chanda D, Roy T, Banerjee K, Munshi S, Patro BS, Chakrabarti S (2011) Mitochondrial dysfunction mediated by quinone oxidation products of dopamine: implications in dopamine cytotoxicity and pathogenesis of Parkinson's disease. Biochim Biophys Acta 1812:663–673. https://doi.org/10.1016/j.bbadis.2011.02.013

Klegeris A, Korkina LG, Greenfield SA (1995) Autoxidation of dopamine: a comparison of luminescent and spectrophotometric detection in basic solutions. Free Radic Biol Med 18:215–222

Lai CT, Yu PH (1997)Dopamine- and L-beta-3,4-dihydroxyphenylalanine hydrochloride (L-Dopa)-induced cytotoxicity towards catecholaminergic neuroblastoma SH-SY5Y cells. Effects of oxidative stress and antioxidative factors. Biochem Pharmacol 53:363–372

Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9:2277–2293. https://doi.org/10.1089/ars.2007.1782

Puspita L, Chung SY, Shim JW (2017) Oxidative stress and cellular pathologies in Parkinson's disease. Mol Brain 10:53. https://doi.org/10.1186/s13041-017-0340-9

Solomon EI, Sundaram UM, Machonkin TE (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2606. https://doi.org/10.1021/cr950046o

Surmeier DJ (2018) Determinants of dopaminergic neuron loss in Parkinson's disease. FEBS J 285:3657–3668. https://doi.org/10.1111/febs.14607

Willems PH, Rossignol R, Dieteren CE, Murphy MP, Koopman WJ (2015) Redox homeostasis and mitochondrial dynamics. Cell Metab 22:207–218. https://doi.org/10.1016/j.cmet.2015.06.006

Zelko IN, Mariani TJ, Folz RJ (2002) Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD(SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33:337–349

Zeng XS, Geng WS, Jia JJ, Chen L, Zhang PP (2018) Cellular and molecular basis of neurodegeneration in Parkinson disease. Front Aging Neurosci 10:109. https://doi.org/10.3389/fnagi.2018.00109

Zorov DB, Juhaszova M, Sollott SJ (2014) Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev 94:909–950. https://doi.org/10.1152/physrev.00026.2013

Zucca FA, Basso E, Cupaioli FA, Ferrari E, Sulzer D, Casella L, Zecca L (2014) Neuromelanin of the human substantia nigra: an update. Neurotox Res 25:13–23. https://doi.org/10.1007/s12640-013-9435-y