Journal of Neural Transmission (2020) 127:213–230
https://doi.org/10.1007/s00702-019-02133-6

NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - ORIGINAL ARTICLE

6-Hydroxydopamine: a far from simple neurotoxin

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Received: 25 November 2019 / Accepted: 21 December 2019 / Published online: 1 January 2020
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Abstract
6-Hydroxydopamine (6-OHDA), which is a neurotoxin that selectively destroys catecholaminergic nerves in sympathetically innervated tissues, has been used to provide a model of Parkinson's disease in experimental animals. It is rapidly autoxidised to yield potentially toxic products and reactive oxygen species. Its ability to release Fe(II) from protein storage sites also results in the formation of hROS. This account will consider how this family of toxic products may contribute to the observed effects of 6-OHDA.

Keywords Neurotoxicity · Aminochrome · Apoptosis · Autoxidation · Dopamine · Highly reactive oxygen species (hROS) · 4-Hydroxynonenal · Mitochondria · Iron release · Necrosis · Oxidative damage · Parkinson's disease · Peroxynitrite · Reactive oxygen species (ROS)

Abbreviations

aCSF	Artificial cerebrospinal fluid
HNE	4-Hydroxynonenal
hROS	Highly reactive oxygen species
MAO	Monoamine oxidase
PBD	Phosphate-buffered saline
6-OHDA	6-Hydroxydopamine
ROS	Reactive oxygen species

Introduction

6-Hydroxydopamine (6-OHDA) is a neurotoxin that selectively destroys catecholaminergic nerves in sympathetically innervated tissues (Thoenen and Tranzer 1968; Ungerstedt 1968). It has been used to provide a model of Parkinson's disease in experimental animals (see, e.g., Deumens et al. 2003; Blandini et al. 2008). It has also been applied in neonatal rats to provide an animal model of Lesch–Nyhan disease (Knapp and Breese 2016) and perinatally for an animal model of attention-deficit hyperactivity disorder (ADHD) (Kostrzewa et al. 2016).

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Despite the fact that the 6-OHDA model has been extensively studied for over five decades, there is still uncertainty about how it works. Different mechanisms have been asserted as the primary basis of its actions by different groups.

- Radicals generated during its breakdown cause extensive damage.
- Reactive oxygen species (ROS) cause non-specific damage.
- It causes the release of transition metals that can then form highly reactive oxygen-containing radicals (hROS) in the Fenton Reaction.
- It, or its breakdown products, react with specific proteins.
- It modifies thiol groups in essential proteins.
- It inhibits mitochondrial function.
- Generalised protein modifications cause an unfolded-protein response.

In reality, all these processes and a few more are likely to result from 6-OHDA administration and their consequential effects may lead through common mechanisms. It appears that any paper claiming to have established a central mechanism of 6-OHDA toxicity will be gainsaid by others championing different processes. These effects and their interrelated outcomes will be considered in this account, which will also incorporate some of our own results, where relevant.

Materials and methods (for section "Stability")

The chemicals used were of the highest available purity and purchased from Sigma-Aldrich Chemical Company. Double distilled or deionized water (resistance ≥ 18.2 mΩ) was used for all preparations. Stock solutions of 6-OHDA HCl were prepared in 0.1 M HCl, since 6-OHDA is unstable in solution at neutral pH values (Sullivan and Stern 1981). Stock solutions of 0.1 M calcium- and glucose-free artificial cerebrospinal fluid (aCSF) were prepared by dissolving 8.17 g (140 mmol) of NaCl, 223.7 mg (3 mmol) of KCl, 203 mg (1.2 mmol) of $MgCl_2$, 213 mg (1.2 mmol) of $Na_2HPO_4 \cdot 2H_2O$ and 37.4 mg (0.27 mmol) of Na_2HPO_4 in 1 l distilled water. The pH was then, if necessary, adjusted to 7.2. For some experiments, glucose (10 mM) was dissolved in the aCSF immediately before use. Stock solutions of $10 \times$ PBS (phosphate-buffered saline) were prepared by dissolving 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4 \cdot 2H_2O$ (F.W. 177.99 g mol^{-1}), and 2.4 g of KH_2PO_4 (F.W. 136.09 g mol^{-1}) in 800 ml of distilled water. This was then brought up to 1 l. The solution was diluted tenfold before use, when the final pH was 7.2. A stock solution of 100 mM terephthalic (TA^{2-}) was prepared by dissolving 2 g in exactly 50 ml of 1 M sodium hydroxide (NaOH). The autoxidation reaction was started by adding a small volume (10–40 µl) of the 6-OHDA solution to 2.5 ml of the buffer solution in a 3 ml quartz cuvette. Data were fitted to a first-order equation by non-linear regression, using the program GraphPad Prism. All determinations were carried out in triplicate and significance of difference was assessed by the Student's *t* test.

Formation of the p-quinone from 6-OHDA was monitored at 490 nm in a Cary 300 UV–Vis spectrophotometer, with 1 cm path-length quartz cuvettes. The molar absorbance coefficient at that wavelength for the product was taken as 1892 l mol^{-1} cm^{-1} (Sachs et al. 1975). The autoxidation of 6-OHDA was studied in 0.1 M potassium phosphate buffer at various pH values. For studies in the presence of sulfhydryl compounds, the absorbance was also monitored at 350 nm, where the thiol conjugate absorbs (Soto-Otero et al. 2000).

Highly reactive oxygen species (hROS) were determined by following the hydroxylation of terephthalic acid fluorometrically (Barreto et al. 1995; Freinbichler et al. 2011) with a Perkin-Elmer LS 55 luminescence spectrophotometer with excitation and emission wavelengths set at 315 and 435 nm, respectively. The excitation slit-width was set at 10 nm with the data interval set at 5 s. TA^{2-} was added to phosphate buffer to the required concentration and after preincubation at 37 °C for 10–15 min, the reaction was started by the addition of 6-OHDA.

Results and commentary

What's in a name?

Most things about 6-hydroxydopamine are confusing, even the proper name for the compound is a matter of contention. The correct IUPAC name is 5-(2-aminoethyl)-1,2,4-benzenetriol, but that is too cumbersome for general use. Oxidopamine has been recommended by some sources, but in that case it seems unclear what 5-hydroxydopamine might be called. The same considerations apply to topamine (trihydroxyphenylethylamine). 2-(2,4,5-Trihydroxyphenyl)ethylamine has the benefit of numbering the substituents correctly and being unambiguous and several variations of this nomenclature have been used, but for the sake brevity, 6-OHDA, as an abbreviation for 6-hydroxydopamine, will be used in this account.

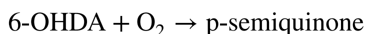
Stability

6-Hydroxydopamine is readily oxidized in solution at neutral pH and it has been claimed that it is so rapidly degraded that there will be little or none left by the time that it is used in normal in vivo or in vitro experiments. Nappi and Vass (1994) reported that essentially all 6-OHDA would be oxidized in 0.5 min in phosphate buffer at physiological pH values. Furthermore, they found that glutathione and ascorbate, which are frequently added in attempts to stabilize the compound were without significant effect on the decay. This rapid autoxidation of 6-OHDA has been confirmed by others, although Soto-Otero et al. (2000); did report some apparent stabilization by ascorbate and thiol compounds.

6-OHDA autoxidation forms the corresponding p-quinone which then cyclises to form a leuco-aminochrome that is then oxidised to an aminochrome, as shown in Scheme 1. The aminochrome can then polymerize to form a neuromelanin. The term aminochrome is imprecise, since it is merely descriptive of the red-coloured material resulting from the oxidation of catecholamine derivatives. It has been applied to several related compounds including ring-substituted derivatives of the two equilibrating species shown in Scheme 1.

Figure 1 shows the time-course of 6-OHDA autoxidation, as monitored by 490 nm. The formation appeared to follow pseudo-first-order kinetics.

For the reaction:

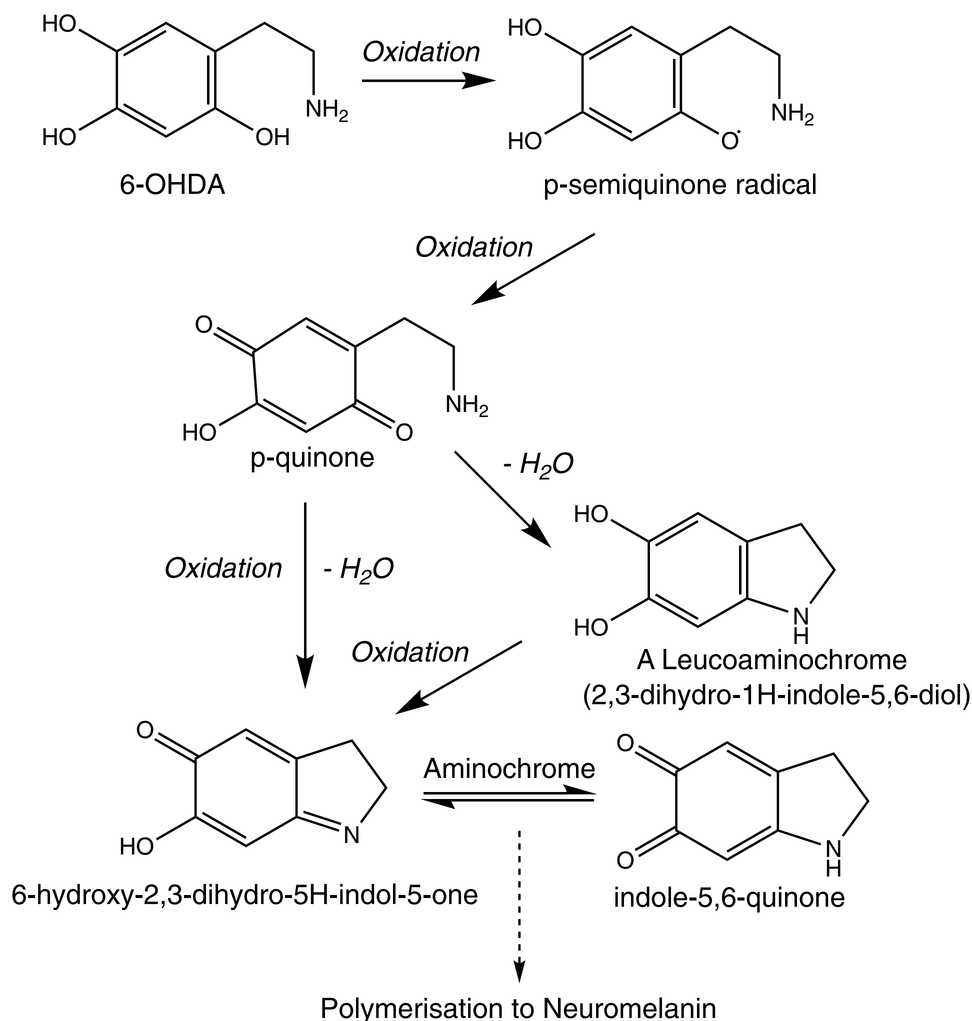


$$\text{6-OHDA} + O_2 \rightarrow \text{p-semiquinone}$$

the first-order equation would be:

$$dp/dt = k'[\text{6-OHDA}]$$

Scheme 1 Simplified autooxidation scheme for 6-OHDA showing the toxic species discussed in the text. The term 'aminochrome' is a non-specific name, which has been aplied to several related species including ring-substitited derivatives of the equilibrating compounds shown



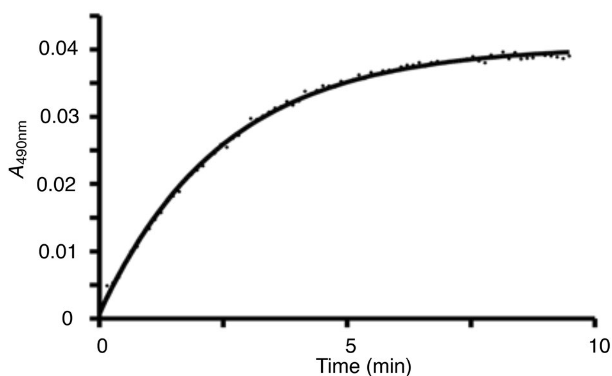


Fig. 1 Time-course of the autoxidation of 25 μM 6-OHDA in 0.1 M phosphate buffer. The data points were fitted to a first-order curve

where the concentration of oxygen is very much greater than that of 6-OHDA so that it remains essentially constant. Under those conditions, the apparent rate constant for the reaction (k') would be:

$$k' = k[O_2]$$

As will be discussed in "Products of 6-OHDA autoxidation", the reaction is, in fact, more complex, with an initial 'priming reaction' generating superoxide that facilitates quinone formation, but this initial process must be too rapid to distort the simple pseudo-first-order dependence.

The rate of autoxidation, gave an apparent first-order rate constant of 0.40 ± 0.06 min^{-1} for the formation of the p-quinone from 25 μM 6-OHDA. This corresponds to a half-life ($T_{1/2}$) = 1.73 ± 0.26 min. Thus about 13.5% of the 6-OHDA will be left after 5 min and there would be less than 2% remaining after 10 min. The total change in absorbance after 10 min would correspond to 84.6% of the 25 μM 6-OHDA having been converted to the quinone. The resulting solution rapidly developed a red colour, corresponding to formation of the aminochrome, which after 48 h had changed into a black pigment, representing melanin formation.

As shown in Table 1, the addition of ascorbate, taurine (10 mM), reduced glutathione, L-cysteine or 1.5 units of catalase had rather small effects on the rate of 25 μM

Table 1 The effects of different additives on autoxidation of 25 μM 6-hydroxydopamine in 0.1 M phosphate buffer, pH 7.2 monitored by determining quinone formation at 490 nm

Addition	k' (min^{-1})	Half-life (min)	% conversion
None	0.400 ± 0.051	1.73 ± 0.26	85 ± 4
Ascorbate (10 mM)	0.358 ± 0.065*	1.94 ± 0.4	64 ± 2
Taurine (10 mM)	0.3541 ± 0.093*	1.97 ± 0.5	56 ± 2
GSH (10 mM)	0.371 ± 0.033*	1.96 ± 0.4	62 ± 0.9
L-Cysteine (10 mM)	0.384 ± 0.082*	1.80 ± 0.39	42 ± 0.2
Catalase (1.5 units)	0.376 ± 0.061*	1.74 ± 0.3	40 ± 0.4
EDTA (1 mM)	0.652 ± 0.094**	1.09 ± 0.16	51 ± 1.3
EDTA + 50 mM Fe(II)	0.898 ± 0.173**†	0.77 ± 0.15	47 ± 0.4
EDTA + Fe(II) + 10 mM Ascorbate	0.433 ± 0.112*†	1.60 ± 0.44	42 ± 2

% conversion values are the calculated percentage after 10 min of that expected if all the 6-OHDA were converted to the p-quinone

*Significantly different from no addition $p > 0.01$

**$p < 0.001$

†Significantly different from the EDTA addition $p < 0.001$

6-OHDA autoxidation, whereas 1 mM EDTA significantly increased the apparent rate constant. Addition of 50 mM $FeSO_4$ to the EDTA gave a further increase, to $0.99 \pm 0.173\ min^{-1}$, whereas addition of 10 mM ascorbate to that mixture resulted in a decrease in the rate constant $0.433 \pm 0.112\ min^{-1}$, indicating some stabilising effect under these conditions. The decreased percentage conversion values in the presence of sulfhydryl compounds can be accounted for by the formation of the S-quinone derivatives, whereas the effects of EDTA may indicate an enhancement of quinone removal.

As might be expected from the need for a priming reaction to initiate autoxidation, the apparent first-order decomposition was neither independent, as would be the case for a truly first-order process, nor linearly dependent on the 6-OHDA concentration (Fig. 2a). The rate of decomposition was also temperature-dependent (Fig. 2a). The apparent first-order rate constant was found to increase linearly with temperature in the range 20–37 °C, with the k' for 50 μM 6-OHDA increasing 3.75 ± 0.15 fold when the temperature was increased from 20 to 37 °C. The rapidity of the process precluded accurate measurements above 37 °C. The effects of pH on the rate of decomposition of 6-OHDA are shown in Fig. 2b. The pK_a value calculated from these data was 7.24. Clearly the compound is quite stable at pH values below 6.0 whereas k' was about three times higher at pH 8 than it was at pH 7.2. This may be an important consideration for studies where 6-OHDA is infused directly into the brain where the pH of the synaptic cleft has been estimated to be about 7.5, falling to about 6.9 during exocytosis (Palmer et al. 2003). This compares with a cytoplasmic pH of about 7.2 in the nerve terminal (Heinonen and Akerman 1986).

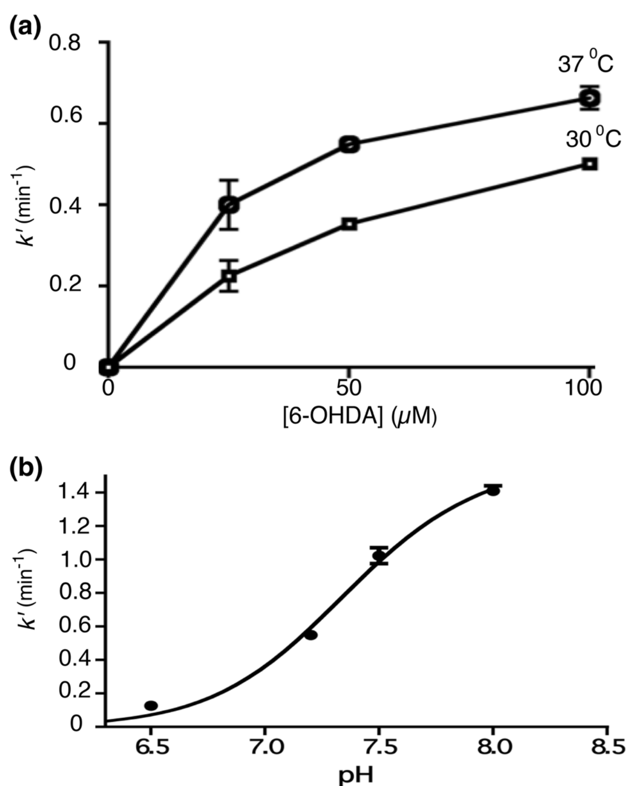


Fig. 2 Effects of temperature (**a**) and pH (**b**) on the apparent first-order rate constant (k') for the autoxidation of 6-OHDA. The pH dependence was determined for 50 μM 6-OHDA. Each point is the mean ± S.E.M. ($n = 3$)

The rapid decay of 6-OHDA (100 μM) in phosphate buffer prompted investigation of its behaviour in alternative media, with the results shown in Fig. 3.

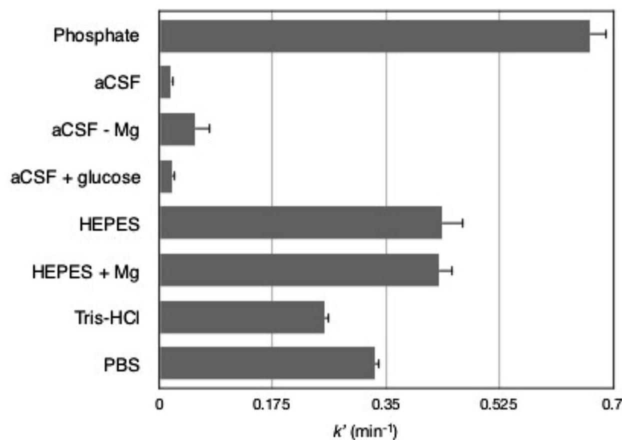


Fig. 3 The effects of different buffer media on the apparent first-order rate constant (k') for the autoxidation of 6-OHDA. The buffer concentrations were 0.1 M. $MgCl_2$ (100 mM) and glucose (10 mM) were added where indicated. Each value is the mean ± S.E.M. ($n = 3$)

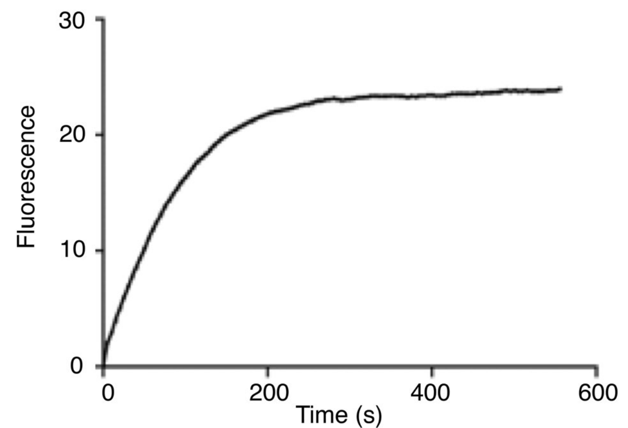


Fig. 4 Fluorescence trace of hROS formation during autoxidation of 25 µM 6-OHDA monitored by reaction with terephthalic acid

Artificial cerebrospinal fluid (aCSF) was used because it was the vehicle for infusions that we have frequently used in microdialysis studies (e.g., Dexter et al. 2011). As can be seen, the use of aCSF had a marked stabilizing effect, in comparison with phosphate buffer. The inclusion of 10 mM glucose, which is usually added to aCSF, had no further effect. Omission of Mg^{2+} from the aCSF resulted in a relatively small increase in the rate, perhaps resulting from it binding to the *ortho*-hydroxyl groups of 6-OHDA (Rajan et al. 1971; Alegría et al. 2004), although addition of Mg^{2+} to the HEPES buffer had no effect. Comparison of phosphate buffer with PBS suggested that ionic strength also had some stabilising effect. Comparison of the effects in Tris–HCl and HEPES with those in phosphate buffer, indicates that phosphate, itself, may stimulate in the autoxidation of 6-OHDA.

The autoxidation of 6-OHDA resulted in a rapid formation of hROS, as shown in Fig. 4. The formation of hROS, which probably resulted from traces of iron contamination, was more rapid and of shorter duration than para-quinine formation. The total change in fluorescence (ΔF) gives a semi-quantitative measure of hROS formation. Increasing the concentration of 6-OHDA increased ΔF from 242 ± 6 at 25 µM to 335 ± 2 and 670 ± 3 at 50 µM and 100 µM 6-OHDA, respectively. As shown in Table 2, ascorbate, GSH and L-cysteine gave significant increases in the half-life and hROS formation, whereas EDTA significantly increased hROS formation without affecting the half-life.

Is it an endogenous compound?

Both 5-and 6-OHDA have been detected in human urine with elevated levels being reported in subjects receiving L-dopa therapy (Andrew et al. 1993). It has also been detected in caudatal biopsy samples from subjects with Parkinson's disease (Curtius et al. 1974) and in mice following long-term L-dopa administration (Borah and Mohanakumar 2010). The possible pathways for the formation of 6-OHDA in vivo are unclear. It can be formed non-enzymatically in a metal-ion catalysed process involving hydroxyl radicals (Borah and Mohanakumar 2009; Jellinger et al. 1995) and by a reaction of dopamine with fatty acid hydroperoxides that is catalysed by iron (Pezzella et al. 1997). It may also be produced enzymatically from the action of polyphenol oxidase, tyrosinase, catechol oxidase, and peroxidase (Hansson et al. 1981; Napolitano et al. 1995). It might also arise from the turnover of the copper-containing oxidases which contain

Table 2 The effects of different additives on hROS formation during the autoxidation of 25 µM 6-hydroxydopamine in 0.1 M phosphate buffer, pH 7.2 monitored fluorometrically by determining the hydroxylation of terephthalic acid

Addition	Half-life (s)	Δ Fluorescence
None	75 ± 10	242 ± 6
Ascorbate (10 mM)	165 ± 4**	805 ± 6**
Taurine (10 mM)	93 ± 2**	224.2 ± 4.1*
GSH (10 mM)	185 ± 6**	558 ± 13**
L-Cysteine (10 mM)	165 ± 3**	364 ± 4.0**
Catalase (1.5 units)	50 ± 5*	274 ± 15*
EDTA (1 mM)	75 ± 3	798.4 ± 2.4**
EDTA + 50 mM Fe(II)	82.4 ± 6	269 ± 8**†
EDTA + Fe(II) + 10 mM Ascorbate	40 ± 5.1**†	741.1 ± 2.3**†
Dithiothreitol (1 mM)	76 ± 4	244 ± 5

Δ Fluorescence values are the total changes in fluorescence (arbitrary units)

*Significantly different from no addition $p > 0.01$

**$p < 0.001$

†Significantly different from the EDTA addition $p < 0.001$

peptide-linked 6-hydroxydopa (TOPA) as an essential cofactor (see Hartmann and McIntire 1997). Although 6-OHDA would be quite stable at the normal urinary pH of about 6, its detection in brain tissue would suggest the presence of stabilizing factors, as discussed above. The possibility of its formation by enzymes in the gut microbiome, where the external pH of the proximal ileum and caecum would favour stability of released 6-OHDA (Maurer et al. 2015), cannot be excluded.

Specificity

Since 6-OHDA cannot pass the blood–brain barrier, its behaviour in vivo has been studied by direct administration into the brain, where some specificity towards dopaminergic nerves may be ensured by careful selection of the site where it is administered, and, in some studies, by the co-administration of a selective noradrenaline-uptake inhibitor, such as desipramine (Luthman et al. 1989). The transporter-mediated accumulation of 6-OHDA in catecholaminergic nerves was demonstrated by Jonsson and Sachs (1971) and confirmed in many subsequent studies (e.g., Cerruti et al. 1993; Storch et al. 2004). Its binding to the transporter in PC12 cells was shown to be competitive with respect to dopamine, with a K_i value of 430 µM, although the inhibition became irreversible over time (Decker et al. 1993). This value is, however, very high compared with the K_m of 209 nM reported for dopamine uptake by the transporter in mouse brain synaptosomes (Ross 1991). Other reports have given K_m values for dopamine that ranged from 67 nM (Enyedy et al. 2003) to 66 µM (Zhang et al. 2009), depending on the experimental conditions as well as, perhaps, the cell type expressing the transporter (see Vaughan and Foster 2013). The importance of the dopamine transporter for selective neurotoxicity was supported by the report that a co-expression of mutant form of α-synuclein of the type found associated with Parkinson's disease increased the sensitivity of kidney cells expressing the dopamine transporter to 6-OHDA, but had no effect on cells that did not (Lehmensiek et al. 2006). That work also reported that the concentration of 6-OHDA necessary to give half maximum toxicity after 24 h (the TC_{50} value) was 88 µM but decreased to 59 µM in the presence of one α-synuclein mutant (A30P) and 38 µM in the presence of another (A53T).

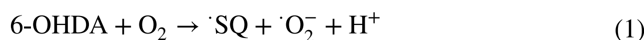
Inhibition of the dopamine transporter was shown to provide partial protection of cultured dopaminergic neurons against 6-OHDA toxicity (Cerruti et al. 1993). The fact that protection was not complete would imply that 6-OHDA may also exert toxic effects by processes that are independent from uptake. Indeed the toxicity of 6-OHDA towards PC12 cells is not affected by inhibitors of catecholamine transport (Hanrott et al. 2006; Blum et al. 2000) and no detectable levels of 6-OHDA were found in cytosolic extracts of PC12 or P19 cells following incubation with up to 600 mM 6-OHDA (Woodgate et al. 1999), suggesting toxicity to be an extracellular process in these cells. Studies with primary cultures of chromaffin cells (Abad et al. 1995), have also shown uptake inhibitors to have only small or no effect on 6-OHDA toxicity. In cultures of dissociated foetal rat mesencephalic cells 6-OHDA was found to be a non-selective neurotoxin, which, at concentrations between 10 and 100 µM, destroyed both dopaminergic and non-dopaminergic cells. Dopamine, itself was also toxic at slightly higher concentrations, with concentrations of 100–300 µM being toxic towards all cell types in the system (Michel and Hefti 1990). A similar indiscriminate toxicity was observed in dissociated-cell cultures from rat cerebral cortex (Rosenberg 1988).

Thus, it appears that low concentrations of 6-OHDA may be selectively toxic towards dopaminergic neurons because of its specific, dopamine-transporter-mediated uptake into the terminals, but higher concentrations act promiscuously in a transporter-independent process. For example, destruction of serotoninergic nerves has also been reported at higher 6-OHDA concentrations (Commins et al. 1989). Studies on the effects of 6-OHDA on neuroblastoma cells in bone marrow were consistent with this dichotomy; showing that unspecific toxicity, which also affected other tumour cells, occurred at higher concentrations of 6-OHDA, but that there was also a specific toxicity at lower concentrations that was dependent on uptake by the neuroblastoma cells (Bruchelt et al. 1985).

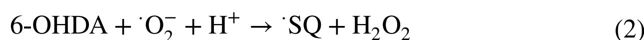
Although it might be feasible to deduce the mechanism of toxicity from the dose of 6-OHDA administered, with low doses acting intracellularly, in a transporter-driven mechanism, and higher doses having an extracellular action, the problem of the instability of 6-OHDA at neutral pH values means that it is difficult to know how much of the compound itself and how much of its oxidation products were actually administered to the target system, as well as the length of time the system was exposed to 6-OHDA itself.

Products of 6-OHDA autoxidation

As shown in Scheme 1, the autoxidation of 6-OHDA results in the formation of the corresponding p-quinone in a process that progresses via the p-semiquinone radical (see, e.g., Heikkila and Cohen 1973; Tiffany-Castiglioni et al. 1982; Padiglia et al. 1997). The system may be represented by the simple scheme that involves alternative reactions:

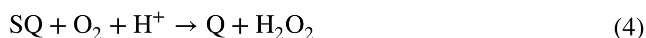


$$\text{6-OHDA} + O_2 \rightarrow {}^{\cdot}SQ + {}^{\cdot}O_2^- + H^+ \quad (1)$$



$$\text{6-OHDA} + {}^{\cdot}O_2^- + H^+ \rightarrow {}^{\cdot}SQ + H_2O_2 \quad (2)$$

$$SQ + O_2 \rightarrow Q + {}^{\cdot}O_2^- + H^+ \quad (3)$$

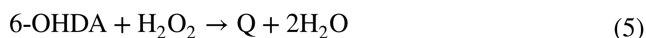


$$SQ + O_2 + H^+ \rightarrow Q + H_2O_2 \quad (4)$$

where $^{\cdot}SQ$ is the p-semquinone radical, SQ the p-semiquinone and $^{\cdot}O_2$ is superoxide.

Reaction (1) can be regarded as a priming process that provides sufficient superoxide for the, more-rapid, subsequent reactions to take place. This has been shown to result in a lag period in the time-course of quinone formation in the presence of superoxide dismutase (Sullivan and Stern 1981). The addition of superoxide dismutase also results in substantial inhibition of quinone formation (Padiglia et al. 1997; Gee and Davison 1989), which is, however, not complete (Soto-Otero et al. 2000). The rate of the reaction process is greatly increased in the presence of Fe(II) and a suitable chelating agent, although the products are unchanged.

In the presence of high concentrations of hydrogen peroxide 6-OHDA may be oxidised directly to the p-quinone:



$$6\text{-OHDA} + H_2O_2 \rightarrow Q + 2H_2O \quad (5)$$

resulting in a cyclic ROS-generating process.

The copper-binding protein ceruloplasmin (ferroxidase; EC 1.16.3.1) has been reported to catalyse the oxidation of 6-OHDA to the p-quinone, without forming the semiquinone intermediate, in a reaction that also produced water rather than hydrogen peroxide (Medda et al. 1996; Floris et al. 2000). It has also been reported that the enzyme tyrosinase (EC 1.14.18.1), which catalyses the oxidation of tyrosine to dopaquinone, may catalyse this reaction (Rescigno et al. 1998). A further complication is the possibility that the o-quinone might be formed from 6-OHDA and subsequently tautomerise to the p-quinone (Graham 1978).

There is evidence for each of the early products of 6-OHDA autoxidation being involved in its toxicity. The p-semiquinone, which is a highly reactive compound, has been suggested to be responsible for 6-OHDA toxicity by Villa et al. (2013), who reported that DTdiaphorase [NAD(P)H dehydrogenase (quinone); EC 1.6.5.2] attenuated 6-OHDA toxicity by reducing the p-quinone directly back to 6-OHDA without the formation of the semiquinone intermediate. The p-quinone, itself, can react with sulfhydryl groups, where the adduct involves substitution at the 2-position of 6-OHDA ring (Liang et al. 1997). A similar reaction occurs with dopaminoquinone (Jameson et al. 2004). This reaction can result in enzyme inhibition and the depletion of the cellular antioxidant defence molecules: glutathione and cysteine. This led to the suggestion that the p-quinone is the causative factor in 6-OHDA toxicity and that addition of glutathione or *N*-acetylcysteine had a protective effect because they remove the quinone before it can react with other targets (Izumi et al. 2005). It has been reported that a thiol group in protein deglycase DJ-1 (Parkinson disease protein 7; a product of the *PARK7* gene), which functions as a transcriptional regulator and protects neurones against oxidative stress is particularly sensitive to the p-quinone leading to its inactivation (Miyama et al. 2011). Since deglycase DJ-1 is believed to respond to the oxidative stress by increasing reduced glutathione (GSH) levels by increasing the transcription of enzymes involved in its synthesis (Shendelman et al. 2004; Zhou et al. 2006), this inhibition will compromise the antioxidant response. The protective role may also involve it inhibiting the aggregation of α-synuclein, after oxidation of a single sulfhydryl group in DJ-1 to the sulfinate (Blackinton et al. 2009).

6-OHDA itself can reduce disulphide bonds resulting in an increased number of SH groups to react with its autoxidation products. Cross-links, producing insoluble aggregates in the cell, can occur a process that involves reaction of the sulfhydryl groups with both the 2 and 5 positions of the p-quinone (Rotman et al. 1976). Alternatively, the 2-S substituted p-quinone group may react with protein amine groups.

Bisaglia et al. (2007) reported that the aminochrome, indole-5,6-quinone, might be the actual toxin, perhaps acting by binding to α-synuclein, which is involved regulating synaptic vesicle recycling and transmitter release. α-Synuclein can also form aggregates, which form the main structural component of Lewy body fibrils in Parkinson's disease (see, e.g., Emamzadeh 2016). However, Lewy bodies, which are a characteristic feature of Parkinson's disease, are not found in experimental models treated with 6-OHDA, probably reflecting the acute nature of its actions as opposed to the progressive age-dependent nature of the disease (see, e.g., Tieu 2011).

As with most other simple explanations of 6-OHDA toxicity, there have been conflicting interpretations, such as the protective actions of GSH and *N*-acetylcysteine simply reflecting their abilities to counteract the effects of ROS toxicity. The autoxidation of 6-OHDA involves the production of the reactive oxygen species hydrogen peroxide and superoxide. The abilities of both these compounds to cause tissue damage at high concentrations, unless they are rapidly removed in reactions catalysed by catalase, the peroxidases and superoxide dismutase, are well documented (see Forman 2007; Gough and Cotter 2011). Thus, *N*-acetylcysteine, which has been shown to protect against 6-OHDA toxicity in several systems, including zebrafish larvae (Benvenutti et al. 2018), might do so by reaction with ROS or retarding the autoxidation of 6-OHDA (Soto-Otero et al. 2000). It would, however, be facile to interpret a fall in reduced GSH to its reaction with ROS or 6-OHDA autoxidation products. For example, 6-OHDA (100 µM) was found to decrease the levels of both GSH and total glutathione in primary cultures of astrocytes after 48 h, which was attributed

to increased levels of the glutathione metabolising enzyme, γ-glutamyltransferase (EC 2.3.2.2) (Zhang et al. 2005), an enzyme that has been reported to be activated by peroxynitrite (Ji and Bennett 2003).

Ascorbate has frequently been used as an antioxidant or to retard 6-OHDA autoxidation. Its effects, however, can be confusing, since it can act as an antioxidant or give rise to a redox-cycling reaction in which it reduces the quinone to the semiquinone radical, which is then oxidised back to the quinone (Pileblad et al. 1988; Roginsky et al. 1998).

The neuromelanin end-product may actually be protective. This seems to be related to its ability to sequester a variety of potentially damaging substances, such as toxic catechol derivatives and redox active transition metals, including iron and copper (Sulzer et al. 2000; Monzani et al. 2019). However, as neurons die the release of neuromelanin may result in neuroinflammation by stimulating glial cells (Zucca et al. 2014). Furthermore, it appears that overproduction of neuromelanin may, itself, be neurotoxic (Carballo-Carbajal et al. 2019).

Iron release

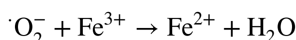
The high reduction potential of 6-OHDA (+ 154 mV at pH 6.8; Graham 1978) is sufficient for releasing iron in its ferrous Fe(II) from the iron-storage protein ferritin (Gerlach et al. 2003; Jameson et al. 2004), as well as from some other proteins, including transferrin (Borisenko et al. 2000) and the un-activated active (3Fe(III)-4S) form of cytoplasmic aconitase (Hayes and Tipton 2002). In contrast 6-OHDA is unable to release iron from the activated (4Fe–4S) form of aconitase or from the haem proteins, cytochrome-*c* and haemoglobin, or from the Fe(II)-sulfur cluster enzyme fumarase and iron-loaded synthetic neuromelanin. The iron release from aconitase ($EC_{50} = 8$ μM), which resulted in the release of 2.75 ± 0.25 mol Fe per mol aconitase in 30 min was followed by a slower loss of SH groups with a complete loss of detectable SH groups after incubation with 400 μM 6-OHDA for 4 h at 37 °C. These effects on aconitase may have important consequences for the behaviour of 6-OHDA when administered in vivo, since the [3Fe–4S] form of aconitase acts as an iron-regulatory protein (IRP1), down-regulating the transcription of ferritin and upregulating the transcription of ferritin receptors (see Lushchak et al. 2014).

This iron release and the observation that iron chelators, of a variety of different types, attenuate 6-OHDA neurotoxicity (Ben-Shachar et al. 1991; Zheng et al. 2005; Dexter et al. 2011), implies that 6-OHDA may mediate its neurotoxicity through release of iron. This iron can then induce the formation of free radicals via the Fenton reaction. The observation that the total iron content of the substantia nigra is increased in 6-OHDA-lesioned rats (Oestreicher et al. 1994) as well as in Parkinson's disease (e.g., Dexter et al. 1991) may be a consequence of this iron mobilisation, or a secondary phenomenon related to attempted damage repair.

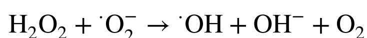
hRos production

Hydrogen peroxide and superoxide can form highly reactive hydroxyl radicals in the presence of transition metals such as Fe(II) through the Fenton and Haber–Weiss reactions

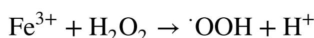
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$



$${}^{\cdot}O_2^- + Fe^{3+} \rightarrow Fe^{2+} + H_2O$$



$$H_2O_2 + {}^{\cdot}O_2^- \rightarrow {}^{\cdot}OH + OH^- + O_2$$



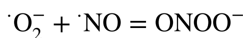
$$Fe^{3+} + H_2O_2 \rightarrow {}^{\cdot}OOH + H^+$$

The generation of hydroxyl radicals in the first reaction is a rather complex process, involving alternative pathways that may lead to Fe(IV)-oxo radical species, crypto radicals as well as $^{\cdot}OH$ (see Freinbichler et al. 2009).

The OH radical is so reactive that it has been estimated to react with a target within 1–5 molecular diameters of its site of formation and its rate of reaction with, for example, linoleate (1 M) of $10^9\ M^{-1}\ s^{-1}$ would correspond to a half-life of 10^{-9} s at 37 °C (see, e.g., Pryor 1986). They cause indiscriminate damage, including base modification and strand breakage in DNA (Ferger et al. 2001a), peroxidation and cleavage in lipids, as well as residue oxidation, hydroxylation and bond cleavage in proteins (Freinbichler et al. 2011; Radi 2018; Sánchez-Iglesias et al. 2007; Ferger et al. 2001b).

Microdialysis studies in which 200 μM 6-OHDA infused into the neostriatum of rats showed a rapid, but transient, release of Fe(II) accompanied by a transient increase in hROS (Freinbichler et al. 2020). The levels of Fe(III) were increased to a greater extent, suggesting the released Fe(II) to be rapidly oxidized, which would be consistent with its involvement of the Fenton reaction, and concomitant iron redox cycling ceasing as 6-OHDA is exhausted (Freinbichler et al. 2009). As might be expected from previous reports (Ben-Shachar et al. 1991; Youdim et al. 2004; Dexter et al. 2011) the inclusion of the iron chelating agent desferoxamine (200 μM) in the perfusion medium, 20 min before 6-OHDA administration, reduced the Fe release to undetectable levels and caused a substantial decrease in hROS. Dajas-Bailador et al. (1998) did not detect any increase in hROS 90 min after intra-striatal injection of 6-OHDA (8 μg), which would be consistent with the transient nature of its formation, although the levels were significantly increased after longer times (120 min and 24 h), suggesting these might be a consequential response to the initial insult.

The superoxide produced during 6-OHDA autoxidation can react with nitric oxide to produce, the highly reactive compound, peroxynitrite (Ferger et al. 2001b; Riobó et al. 2002)



$^{\cdot}O_2^- + {}^{\cdot}NO = ONOO^-$

This can result in nitration of proteins (Riobó et al. 2002; Henze et al. 2005) and DNA damage (Szabó and Ohshima 1997). For convenience the term hROS will be assumed to include reactive nitrogen species in the remainder of this account.

Lipid peroxidation may lead to the production of toxic aldehydes, in particular 4-hydroxynonenal (HNE) is formed during peroxidation of membrane-derived ώ-6 polyunsaturated fatty acids, such as linoleic and arachidonic acids, may act as a signalling molecule at low concentrations (Zhang and Forman 2017) but is neurotoxic at higher concentrations, probably acting through adduct formation with sulfhydryl groups resulting, among other effects in impaired dopamine transport and vesicular storage (Lopachin et al. 2009). It is metabolised by glutathione S-transferases (EC 2.5.1.18) (Singhal et al. 2015), which will further contribute to the loss of sulfhydryl antioxidant defence and decreased synaptosomal glutathione levels. The unsaturated aldehyde acrolein (propenal) is another product of lipid peroxidation (Uchida et al. 1998), which is highly toxic, it binds to proteins and DNA, disrupts mitochondria and damages membranes as well as causing, yet more, oxidative stress (Moghe et al. 2015). In dopaminergic neurons, it has been shown to bind to α-synuclein and cause it to aggregate and to cause apoptotic and necrotic cell death (Wang et al. 2017).

Effects on mitochondria

6-OHDA has been shown to inhibit the mitochondrial electron-transport chain, at the level of Complex I (NADH-ubiquinone reductase) and also at the final Complex IV (cytochrome-*c* oxidase) step (Glinka and Youdim 1995; Glinka et al. 1996; Iglesias-Gonzálezet al. 2012). Dopamine itself has also been shown also to inhibit complex I, albeit at higher concentrations but without affecting Complex IV activity (Ben-Shachar et al. 2004).

The inhibition of complex I in isolated brain mitochondria by 6-hydroxydopamine is reversible and partially uncompetitive in nature with a K_i value of 51 ± 14 μM. Since the antioxidant lipoic (thioctic) acid had no significant effect on the inhibition whereas Fe(III), which might be expected to enhance the autoxidation of 6-OHDA, decreased the inhibition of complex I, it was concluded that the inhibition was due to 6-OHDA itself rather than an autoxidation product. The effects of this inhibition may be more marked in the nerve terminals, where the mitochondrial electron-transport chain has been shown to be more sensitive to complex I inhibition than whole brain mitochondria (Pathak and Davey 2008; Telford et al. 2009) and also to be more sensitive to oxidative damage (Hill et al. 2018). Intriguingly, it appears that this sensitivity to inhibition by 6-OHDA may be a confined to striatal neurons, since studies with rat brain slices (Gonçalves et al. 2019) showed that inhibition was apparent in striatal slices but not in those from the cortex or hippocampus after incubation with 100 μM 6-OHDA for 60 min. These observations suggest that the mitochondria from the other regions were either less sensitive, or more capable of recovery from the effects of 6-OHDA than those in the striatum. In that work, however, *N*-acetyl cysteine was found to protect against these longer-term effects of 6-OHDA, which was interpreted as indicating the involvement of ROS.

Inhibition of mitochondrial electron transport will have several consequences, including a fall in ATP levels, a rise in lactate as 'anaerobic' glycolysis attempts to compensate, and intramitochondrial production of superoxide radicals, which through the action of the superoxide dismutases, releases H_2O_2 (see Murphy 2009). The decreased ATP levels will result in impairment of the synaptic-vesicle proton pump leading to leakage of dopamine into the terminals where it may be oxidised by monoamine oxidase (MAO) producing even more H_2O_2 (see Tipton 2018). Thus, there should be an abundance of substrates for the Fenton and Haber–Weiss reactions to produce nasty radicals. The shortage of ATP will also result in loss of Ca^{2+} homeostasis and failure of the Na^+/K^+ ATPase leading to release of dopamine from the terminals, when it may be metabolised by MAO in glial cells. Direct inhibition of this ATPase by dopamine-autoxidation products has also been reported (Khan et al. 2003).

If these effects were not sufficient to make the dopaminergic cells extremely unhappy, the oxidative stress and disruption of Ca^{2+} homeostasis resulting from complex I inhibition will cause the mitochondrial permeability-transition pore and related structures to open, leading mitochondrial swelling, release of cytochrome-*c*, caspase activation and, depending on the severity of the conditions, apoptosis or necrosis (see Zamzami et al. 2005, Kinnally et al. 2011; Dorn 2013). In addition, an autophagic response has been detected in rats after striatal injection of 15 μg 6-OHDA (He et al. 2017).

The autoxidation of the released dopamine will, presumably, also occur in the nerve terminal and the quinones resulting from this have been reported to cause mitochondrial depolarisation and opening of the permeability-transition pore (Biosa et al. 2018). It has been reported that the cell death may involve activation of the Ras/Raf/extracellular signal-regulated kinase (ERK) signalling pathway in mitochondria (Kulich et al. 2007), which can result in apoptosis or autophagy (see Cagnol and Chambard 2010).

Complex I has also been found to be depressed in Parkinson's disease (Schapira et al. 1989), as are some other respiratory-chain complexes (Chen et al. 2019). The neuronal death in that disease may be apoptotic with some autophagic contribution (Liu et al. 2019).

The inhibition of Complex I by 6-OHDA raises a direct comparison to the pro-neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which readily penetrates the brain, where it is oxidised by extra-neuronal MAO-B to the 1-methyl-4-phenylpyridinium ion (MPP^+). This is then transported into the nerve terminals by the dopamine transporter where it inhibits mitochondrial complex I, resulting in nerve death (see Tipton and Singer 1993). Thus, it is an attractive idea that these quite different, parkinsonism-inducing, toxins operate through similar basic mechanisms (Blum et al. 2001). However, Choi et al. (1999) showed that, in a mesencephalon-derived dopaminergic neuronal cell line (MN9D cells), the processes of cell death induced by the toxins to be different, with cell death being apoptotic following 6-OHDA and involving on ROS-induced pathways, whereas MPP^+ caused necrotic cell death in a mechanism that appeared to be independent from ROS. Lotharius et al. (1999) confirmed 6-OHDA induced cell death to be apoptotic and to be caused by ROS in mesencephalic-cell cultures and that antioxidants could cause complete protection from 6-OHDA but only partial protection against MPP^+ toxicity.

Mazzio et al. (2004) reported that 250 µM 6-OHDA inhibited both anaerobic glycolysis and mitochondrial oxygen consumption in neuroblastoma (2A) cells, but the anaerobic pathway could be restored by the addition of catalase to remove H_2O_2. They attributed the mitochondrial inhibition to 6-OHDA maintaining cytochrome-*c* in its reduced (Fe(II)) state, thereby inhibiting complexes II and III activities. In contrast, Storch et al. (2000) reported that whereas MPP^+ caused a drop in ATP levels at a dose that caused cell death to SH-SY-5Y cells after 24 h, but 6-OHDA (IC50 = 25 µM) did not, leading them to conclude that 6-OHDA toxicity was not due to an inhibition of mitochondrial energy supply, but probably involved production of free radicals. They confirmed this conclusion by showing that α-tocopherol reduced the toxicity of 6-OHDA in this system. A comparison of the effects of 6-OHDA, the complex 1 inhibitor rotenone and MPP^+ in SH-SY-5Y cells also concluded that the main mechanism of 6-OHDA toxicity was not dependent on bioenergetic impairment (Giordano et al. 2012).

Although this account has concentrated upon the interactions of 6-OHDA with mitochondria, the ROS and hROS resulting from its autoxidation can also inhibit components of the electron-transport chain (Zhang et al. 1990) and peroxynitrite also damages mitochondrial function at the levels of complex I and II (Radi et al. 2002).

Modification of proteins

As discussed above 6-OHDA is a powerful reducing agent and as such it may reduce disulphide bonds in proteins. The products of 6-OHDA autoxidation are, however, oxidising agents capable of oxidising –SH groups in proteins to the corresponding sulfenic and sulfinic acids and, perhaps, as far as sulfonates (Gupta and Carroll 2014). Such effects can result in alterations (e.g., Couée and Tipton 1991) or loss of function of thiol-containing enzymes (e.g., Knight and Mudd 1984). Since the 6-OHDA-derived p-quinone can react directly with SH groups to produce –S-6-OHDA adducts, the simple determination of SH groups, such as by determining the release of 5-thio-2-nitrobenzoic acid (Nbs–) from 5,5′-dithiobis(2-nitrobenzoic acid) by the spectrophotometric method of Ellman (1959), will not reveal whether the 'lost' thiol groups resulted from adduct formation or their oxidation to their sulfenates, sulfinates or sulfonates. The loss of sulfhydryl groups in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and consequent loss of its enzyme activity when it was incubated with 6-OHDA was time-dependent with an IC_{50} value of 50.19 ± 0.25 µM (Hayes and Tipton 2002). Inhibition was reversed after 5 min incubation by dithiothreitol or arsenite, suggesting that oxidation to sulphenate had occurred, but it became irreversible after longer periods consistent with further oxidation to the sulfinate or sulfonate. The loss of enzyme activity is consistent with the reactive active-site thiol group which is essential for dehydrogenase activity, having been oxidised. Interestingly, incubation of GAPDH with 6-OHDA did not appear to prevent its binding to single-stranded DNA, except at unfeasibly high concentrations (1 mM). Clearly, the oxidative inhibition of this enzyme would impair any ability of anaerobic glycolysis to compensate for effects of 6-OHDA inhibiting mitochondrial oxidative phosphorylation, but it may have more far-reaching implications since GAPDH also functions in DNA repair and apoptosis and sulfhydryl oxidation appears to enhance these actions (see Chuang et al. 2005; Hwang et al. 2009; Huang et al. 2009). Peroxynitrite has also been shown to inactivate this enzyme by oxidizing the essential sulfhydryl group, but in this case the resulting inhibition was not reversed by arsenite (Souza and Radi 1998).

A number of other enzymes have been shown to be sensitive to inhibition by ROS, including the glycolytic enzyme pyruvate kinase (Anastasiou et al. 2011) as well as 2-oxoglutarate dehydrogenase and mitochondrial aconitase, which are both essential in the tricarboxylic acid cycle (Tretter and Adam-Vizi 2000).

The reaction of deglycase DJ-1 with the 6-OHDA p-quinone has been discussed in "Products of 6-OHDA autoxidation". For molecules that escape this reaction, oxidation of the reactive sulfhydryl group to the sulfinate will activate its antioxidant effects (Blackington et al. 2009), but inactivation

might result if further oxidation to the sulfonate were to occur.

Enzymes involved in dopamine metabolism

Tyrosine hydroxylase, the first enzyme in the metabolic pathway leading to dopamine synthesis, is inactivated by peroxynitrite in a process that involves the oxidation of cysteine residues and the nitration of several tyrosine residues in the enzyme. It appears that the cysteine oxidation is largely responsible for the inhibition (Blanchard-Fillion et al. 2001; Kuhn et al. 2002). 6-OHDA has been reported to be substrate for the soluble form of human catechol-*O*-methyltransferase (Taskinen et al. 2003). If that were the case, methylation would be expected to yield 3-methoxy-4,6-dihydroxydopamine, which would effectively prevent p-quinone formation. However, 6-OHDA, or one of its autoxidation products, has also been shown to be an inhibitor of the enzyme (Borchardt et al. 1976; Reid et al. 1986).

Of the two monoamine oxidase forms, MAO-A predominates in the dopaminergic nerve terminals, whereas MAO-B is present in glial cells. Since dopamine is a good substrate for both forms of MAO (see Tipton 2018), it would be reasonable to consider whether the enzymes also oxidises 6-OHDA. However, despite speculation that this might be the case, there is no convincing evidence for it occurring. In fact hydroxyl radicals have been shown to inhibit both MAO-A and -B irreversibly, with MAO-B being somewhat more sensitive (Soto-Otero et al. 2001). Although that would prevent ROS generated from the MAO-catalysed oxidation of 6-OHDA released from synaptic vesicles, it would still leave the terminals exposed to the toxicity of dopamine itself and its autoxidation products.

Dopamine oxidation by any MAO that escapes inhibition, will produce the corresponding aldehyde, dopal (3,4-dihydroxyphenylacetaldehyde), as well as H_2O_2 (see Tipton 2018). The aldehyde dehydrogenases are the main enzymes metabolising dopal, with the aldehyde reductases playing a minor role (Turner et al. 1974). Aldehyde dehydrogenases contain active-site sulfhydryl groups that are essential for action (Stoppani and Milstein 1957). Thus, these enzymes are inhibited by 6-OHDA autoxidation products (Jinsmaa et al. 2009), resulting in the accumulation of dopal, which has also been reported to occur in the substantia nigra pars compacta of Parkinson's disease subjects (Masato et al. 2019). Dopal is a toxic molecule that can react with the amine groups of lysine in proteins including α-synuclein, which oligomerises leading to nerve terminal damage (Plotegher et al. 2017). Dopal can also react with unchanged dopamine to form tetrahydropapaveroline, which may, itself, be toxic (see Tipton 2018).

Despite the extraneuronal location of MAO-B, a number of inhibitors that are selective towards that form of the enzyme, including L-deprenyl (Selegiline) (Salonen et al. 1996), rasagiline (Azilect) (Blandini et al. 2004) and PF 9601N [*N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] (Cutillas et al. 2002), have been shown to protect against 6-OHDA toxicity. Since these compounds are all propargylamine derivatives, these effects may, at least in part, result from the neuroprotective/neuro-rescuing actions of such compounds, which have been shown to be independent of their MAO inhibitory actions (see Tatton et al. 2003; Inaba-Hasegawa et al. 2017). The rasagiline metabolite aminoindan, which is not an effective MAO inhibitor, also protects against 6-OHDA toxicity (Bar-Am et al. 2007), indicating that other mechanisms must be involved, and there have been many suggestions as to what these might be (see Dimpfel and Hoffmann 2011; Ou et al. 2009; Ledreux et al. 2016). Safinamide, a reversible MAO-B inhibitor and sodium channel-blocker, which like L-deprenyl and rasagiline (Riederer and Laux 2011) has been used in the treatment of Parkinson's disease (Teixeira et al. (2018), has also been shown to protect against 6-OHDA toxicity (Sadeghian et al. 2016).

The β-carboline harmaline, which is a reversible MAO-A inhibitor was shown to afford some protection against 6-hydroxydopamine in PC-12 cells, but harmalol which was a less potent inhibitor (Herraiz et al. 2010) behaved quite similarly (Kim et al. 2001). It was concluded that the protection was owing to the ROS-scavenging actions of these compounds. Not all MAO-A inhibitors protect against 6-OHDA toxicity, since the reversible inhibitor moclobemide, which has been used to counter the depression associated with Parkinson's disease (see Riederer and Laux 2011), appeared to have little or no effect on the rotational behaviour of rats 6 weeks after of 6-OHDA administration, but, as might be expected, it enhanced the effects of L-dopa (MacInnes and Duty 2004). The older, non-selective and irreversible MAO inhibitor phenelzine ([2-phenylethyl]hydrazine) has also been shown to protect against oxidative stress but this appears to result from it acting as a scavenger of toxic aldehydes, such as HNE and acrolein (Baker et al. 2019).

Consequential responses

The initial damage resulting from 6-OHDA can lead to a number of cell death responses. These may involve apoptosis, necrosis, autophagy or catastrophic cell rupture (see Dorn 2013; Lossi et al. 2015; D'Arcy 2019). The response appears to involve the cell attempting to follow a controlled death pathway only to find that the conditions are too serious for that. Caspases of different types will be activated, but Ochu et al. (1998) showed that, although a non-specific caspase inhibitor prevented 6-OHDA-induced apoptosis, it did not protect against necrosis. There will be unfolded-protein responses (Holtz and O'Malley 2003), including increased

ubiquitin conjugation (Elkon et al. 2001). The oxidative stress will result in an inflammatory response, which is also seen in Parkinson's disease (Guo et al. 2018; Wang and Michaelis 2010). The rupture of the cells will, of course, lead to proliferation of microglia and immune responses (see, e.g., Theodore and Maragos 2015; Martinez and Peplow 2018), which will be met by NFκB activation (Youdim et al. 1999; Park et al. 2004). While this is all going on, further nasty events may contribute to this witches' brew; HNE and acrolein, resulting from lipid peroxidation, will damage proteins and interfere with vesicle dopamine storage, leading to nerve death (Lopachin et al. 2009). If MAO survives 6-OHDA toxicity, the oxidation of dopamine released from the vesicles may be sufficient to cause neurotoxicity (see e.g., Norenberg et al. 2004). The disturbed calcium homeostasis resulting from mitochondrial impairment may also lead to calpain-mediated neuronal death (Cheng et al. 2018).

All this mayhem will, of course, lead to changes in protein expression as the system struggles to cope. Microarray expression studies have revealed that a large number of transcripts are affected (Holtz et al. 2005; Park et al. 2011). Analysis of the time-course of changes in expression in cells from the murine mesencephalic cell line, (MN9D) exposed to 6-OHDA in the range 10–100 µM, indicated that the unfolded protein response, resulting from ROS-inflicted damage, acting through the mitochondrial cytochrome-*c* release and consequent caspase activation was the primary process triggering cell death (Holtz et al. 2006).

Conclusions

Examination of the reported responses to 6-OHDA reveals a number of factors that may underlie the confusing literature. Cells or systems that do not contain active dopamine transporters can be killed by extracellular processes, presumably resulting from ROS and hROS formation. Those with active dopamine transporters can suffer intracellular insult that can lead to death responses, but extracellular damage is also likely to occur at the same time. The concentration of 6-OHDA that is administered may be an important factor, but as discussed above, the instability of the compound at physiological pH values makes it difficult to be sure how much of the compound rather than its autoxidation products was actually administered or what the intracellular concentration of 6-OHDA might be at any given time after administration. Estimates of its toxic concentrations, reported in the literature, range from the low micromolar to the millimolar range. There also seems to be quite a narrow difference between the concentrations of 6-OHDA that cause apoptosis and necrosis, for example Ochu et al. (1998) found that, in PC12 cells, 25 µM 6-OHDA caused apoptotic cell death, whereas that caused by 50 µM was mainly necrotic.

The value of the use of 6-OHDA to provide a model of Parkinson's disease is limited by the fact that it is an acute toxin whereas the disease is a relatively slowly developing condition. Thus, although it may be useful in studies of, for example, the behavioural consequences of the loss of nigrostriatal dopaminergic neurons, it may do no more than suggest processes that may be involved in the development of the idiopathic disease.

A quick search of PubMed for "6-hydroxydopamine" reveals that it has generated over 12,500 papers since 1963. It would be impossible to do justice to them all in this short review and we apologize to anyone who feels that their contributions have not been given adequate consideration. However, amongst the data there is general consensus that chelating agents and antioxidants afford some measure of protection against 6-OHDA toxicity, which would be consistent with the importance of ROS and hROS as well as the early products of 6-OHDA autoxidation. The myriad of nasty consequences that will then follow are likely to occur on similar time-scales, such that it may be fruitless to pinpoint one particular damage as being central. It is akin to 50 Roman senators stabbing Julius Caesar and asking who struck the fatal blow—the answer might be that they all may have contributed.

Acknowledgements We are grateful to the Science Foundation Ireland for support. KFT is grateful to Professor Moussa Youdim, for many wide-ranging and stimulating discussions.

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