AOP ID and Title:

SNAPSHOT

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AOP 3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits

Short Title: Mitochondrial dysfunction and Neurotoxicity

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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	EAGMST Under Review	1.33	Included in OECD work plan

Abstract

This AOP describes the linkage between inhibition of complex I (CI) of the mitochondrial respiratory chain and motor deficit as in parkinsonian disorders. Binding of an inhibitor to complex I has been defined as the molecular initiating event (MIE) that triggers mitochondrial dysfunction, impaired proteostasis, which then cause degeneration of dopaminergic (DA) neurons of the nigro-striatal pathway. Neuroinflammation is triggered early in the neurodegenerative process and exacerbates it significantly. These causatively linked cellular key events result in motor deficit symptoms, typical for parkinsonian disorders, including Parkinson's disease (PD), described in this AOP as an Adverse Outcome (AO). Since the release of dopamine in the striatum by DA neurons of the Substantia Nigra pars compacta (SNpc) is essential for motor control, the key events refer to these two brain structures. The weight-of-evidence supporting the relationship between the described key events is based mainly on effects observed after an exposure to the chemicals rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), i.e. two well-known inhibitors of complex I. Data from experiments with these two chemicals reveal a significant concordance in the dose-response relationships between the MIE and AO and within KEs. Also essentiality of the described KEs for this AOP is strong since there is evidence from knock out animal models, engineered cells or replacement therapies that blocking, preventing or attenuating an upstream KE is mitigating the AO. Similarly, there is proved experimental support for the KERs as multiple studies performed with modulating factors that attenuate (particularly with antioxidants) or augment (e.g. overexpression of viral-mutated α-synuclein) a KE up show that such interference leads to an increase of KE down or the AO. Information from in vitro and in vivo experiments is complemented by human studies in brain tissues from individuals with sporadic Parkinson's disease (Keeney et al., 2006) to support the pathways of toxicity proposed in this AOP.

Summary of the AOP

Stressors

Name	Evidence
1',2'-dihydrorotenone	Strong
MPP+	Strong

1',2'-dihydrorotenone

MPP+

Molecular Initiating Event

Title	Short name
Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I) (https://aopwiki.org/events/888)	Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I)

888: Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I) (https://aopwiki.org/events/888)

Short Name: Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I)

AOPs Including This Key Event

AOP ID and Name	Event Type
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	MolecularInitiatingEvent

Stressors

Name
1',2'-dihydrorotenone

Biological Organization

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

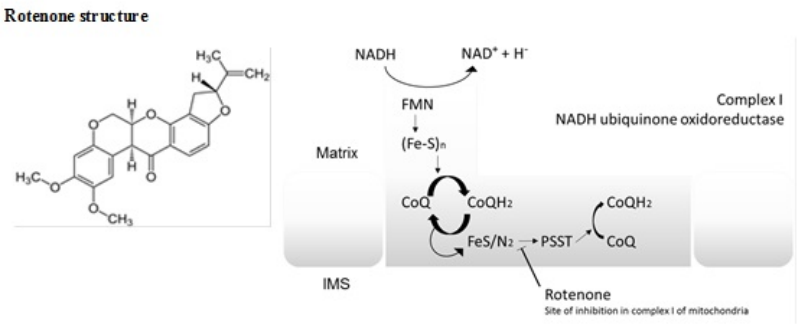
The most studied examples of chemicals that inhibit CI are: rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Desplats et al., 2012; Lin et al., 2012; Sava et al., 2007). Both, rotenone (pesticide) and MPP+ (the active metabolite of MPTP) are well known to reproduce the anatomical, neurochemical, behavioural and neuropathological features of PD-like syndrome (Betarbet et al., 200; Greenamyre et al., 2001). Indeed, an overwhelming evidence has accumulated in the existing literature suggesting such a link and therefore these two inhibitors of CI will be discussed in the context of all KE identified in this AOP.

1. Rotenone affinity to complex I binding sites.

Rotenone (a flavonoid, extracted from the several plants e.g. Derris scandens)is one of the most powerful, an irreversible inhibitor of CI, binding with high affinity to CI and is typically used to define the specific activity of this complex. Rotenone is extremely lipophilic, it crosses biological membrane easily and it gets into brain very rapidly. The interaction of rotenone with active ('pulsed') and thermally de-activated ('resting') membrane-bound Complex I as revealed by inhibition of NADH-ubiquinone- and ubiquinol-NAD+ reductase activities was studied. Ki = 1 x 10(-9) M, k(on) = 5 x 10(7) M-1 min-1 and k(off) = 0.02 min-1 (inhibitory effect of rotenone on NADH oxidation) and Ki = 2 x 10(-8) M (inhibition of reverse electron transfer) were determined for pulsed enzyme. The equilibrium between de-activated and active enzyme is reached (K approximately 100) after the slow strongly temperature-dependent de-activation process has completed. Rotenone partially prevents and reverses the

enzyme de-activation. About two order of magnitude difference in affinity of rotenone to the active and de-activated forms of the enzyme was demonstrated (Grivennikova et al., 1997). Dose-dependent relative affinities of rotenone to the inhibitory site of CI is shown in Fig. 3B (for more detail Grivennikova et al., 1997).

Most of the studies suggest that hydrophobic inhibitors like rotenone or Piericidin A most likely disrupt the electron transfer between the terminal Fe-S cluster N2 and ubiquinone (Fig. 3A).



(https://aopwiki.org/wiki/index.php/File:MIE_Fig._3A.jpg)

Fig. 3A. Rotenone structure and a schematic representation of its binding site (and other Rotenone-like compounds) to CI. IMS: inter-membrane space (based on Lummen, 1998)

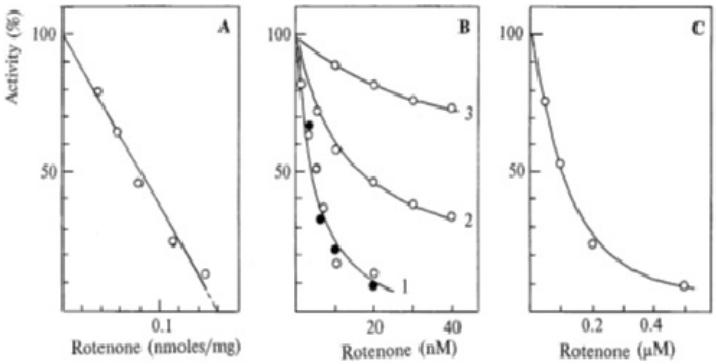


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Fig. 3B. Fig. 2. Relative affinities of rotenone to the inhibitory site(s) of Complex I. Panel (A): activated submitochondrial particles (SMP) (2.8 mg/ml, approx. 0.4 microM Complex I) were incubated in the standard reaction mixture for 20 min at 25oC and residual initial rate of NADH oxidation was measured. 100% correspond to the specific activity of 1.0 micromol/min per mg of protein. Panel (B): curve 1 (o), SMP (48 microg/ml, approx. 8 nM Complex I) were activated in the assay cuvette and pre-incubated with rotenone in the presence of gramicidin and 10 mM malonate for 20 min at 25oC and the residual NADH oxidase activity was then measured; black circle: the same as (o), except that pre-incubation with rotenone was made in the presence of 10 mM succinate (no gramicidin and malonate), 10 mM malonate and gramicidin were added simultaneously with 100 microM NADH to measure the residual activity. Curve 2, presents the reverse electron transfer activity and curve 3, de-activated SMP were preincubated with rotenone as described for curve 1(o) (for further details see Grivennikova et al., 1997). Panel (C): The same as Panel B, curve 3, except for enzyme concentration was 0.5 mg/ml and rotenone concentration range which was increased to show interaction of the inhibitor with de-activated enzyme. The activity was measured after 200-fold dilution into the assay mixture. All the continues lines corresponds to the theoretical titration curves for the reversible single site inhibition with Ki values of 1 nM, 20 nM and 80 nM for the curves 1, 2 and 3, respectively (for further details see *Grivennikova et al., 1997*).

2. MPTP affinity to complex I binding sites. MPTP is not directly binding to CI and it is therefore non-toxic to DA neurons. MPTP exerts its toxicity after it is metabolized by mono-amino-oxidase, type B (MAO B), in astrocytes to 1-methyl-4-phenylpyridinium (MPP+). This metabolite binds to CI, and is toxic. MPP+ is a good substrate for dopamine transporters (DAT), expressed selectively by DA neurons (Greenamyre et al (2001). Due to both a positive charge and an amphoteric character, MPP+ specifically accumulates in mitochondria, where despite a lower affinity to the binding site of complex I than rotenone, it reaches high enough intra-mitochondrial

concentrations to inhibit CI activity (Ramsay et al., 1991). The binding affinity of MPP+ is low (mM range), and it can be totally reversed by washing out. However, prolonged treatment results in a severe, progressive and irreversible inhibition of complex I, most likely by indirect mechanisms involving oxidative damage (Cleeter et al., 1992). Competitive binding experiments with rotenone and MPP+ suggest that the two compounds bind to the same site of the CI (Ramasay et al., 1991).

3. General characteristics of other complex I inhibitors There is a variety of CI inhibitors, both naturally occurring besides rotenone such as Piericidin A (from Streptomyces mobaraensis), acetogenins (from various Annonaceae species) as well as their derivatives, and synthetically manufactured compounds like pyridaben and various piperazin derivatives (Ichimaru et al. 2008). They have been used to probe the catalytic activity of complex I especially in order to clarify its ubiquinone binding site and indeed, most of these compounds inhibit the electron transfer step from the Fe-S clusters to ubiquinone (Friedrich et al. 1994). Therefore, classification of CI inhibitors is based on their types of action. Type A inhibitors, like piericidin A, 2-decyl-4-quinazolinyl amine (DQA), annonin VI and rolliniastatin-1 and -2, are considered to be antagonists of the ubiquinone substrate. For piericidin A, it has been shown that it inhibits NADH:Q2 activity in a partially competitive manner. Contrary to type A, type B inhibitors, like the commonly used rotenone, have hydrogen-bonding acceptors only in the cyclic head of the molecule and are non-competitive towards UQ (ubiquinone), but are believed to displace the semiquinone intermediate during the catalysis (Fig. 2). Finally, inhibitors classified as type C, like stigmatellin and capsaicin, form a third group of hydrophobic CI inhibitors that are believed to act as antagonists of reduced ubiquinone (Degli Esposti 1998, Friedrich et al. 1994, Haefeli 2012) (Fig. 2). Competition studies with representatives of all three different types of inhibitors revealed that type A and B and type B and C, but not type A and C, compete with each other for binding. This led to a suggestion that all CI inhibitors acting at the ubiquinone binding pocket share a common binding domain with partially overlapping sites (Okun et al. 1999).

Some inhibitors bind to the outside of the ubiquinone reduction site and do not fit the preceding classification. Examples of such compounds are ADP-ribose, which competes for substrate binding at the NADH site (Zharova and Vinogradov, 1997), and diphenyleneiodonium (DPI) that covalently binds to reduced flavin mononucleotide (FMN) in the hydrophilic part of the enzyme blocking the electron transfer to the Fe-S clusters (Majander et al., 1994). There are also new, commercially available insecticides/acaricides with potential to inhibit mitochondrial respiration such as benzimidazole, bullatacin, 6-chlorobenzothiadiazole, cyhalothrin, Fenazaquin Fenpyroximate, Hoe 110779, Pyridaben, Pyrimidifen, Sandoz 547A, Tebufenpyrad and Thiangazole (Greenamyre et al., 2001). It is clear that they are capable of inhibiting the mammalian CI of mitochondrial respiratory chain, by binding to and blocking ubiquinone-dependent NADH oxidation with high efficacy (Lummen, 1998).

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

CI has a highly conserved subunit composition across species, from lower organisms to mammals (Cardol, 2011). Fourteen subunits are considered to be the minimal structural requirement for physiological functionality of the enzyme. These units are well conserved among bacterial (E. coli), human (H. sapiens), and Bovine (B. taurus) (Vogel et al., 2007b; Ferguson, 1994). However, the complete structure of CI is reported to contain between 40 to 46 subunits and the number of subunits differs, depending on the species (Gabaldon 2005; Choi et al., 2008). In vertebrates CI consists of at least 46 subunits (Hassinen, 2007), particularly, in humans 45 subunits have been described (Vogel et al, 2007b). Moreover, enzymatic and immunochemical evidence indicate a high degree of similarity between mammalian and fungal counterparts (Lummen, 1998). Mammalian CI structure and activity have been characterized in detail (Vogel et al., 2007a; Vogel et al., 2007b), referring to different human organs including the brain. There is also a substantial amount of studies describing CI in human muscles, brain, liver, as well as bovine heart (Janssen et al., 2006; Mimaki et al. 2012) (Okun et al., 1999).

How this Key Event Works

Electron transport through the mitochondrial respiratory chain (oxidative phosphorylation) is mediated by five multimeric complexes (I–V) that are embedded in the mitochondrial inner membrane (Fig. 1). NADH-ubiquinone oxidoreductase is the Complex I (CI) of electron transport chain (ETC). It is a large assembly of proteins that

spans the inner mitochondrial membrane. In mammals, it is composed of about 45-47 protein subunits (human 45) of which 7 are encoded by the mitochondrial genome (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) and the remainder by the nuclear genome (Greenamyre, 2001). CI oxidizes NADH elevating the NAD+/NADH ratio by transferring electrons via a flavin mononucleotide (FMN) cofactor and several iron-sulfur centers to ubiquinone (Friedrich et al., 1994) (Fig. 1). Binding of an inhibitor to CI inhibits the NADH–ubiquinone oxido-reductase activity, i.e. blocks the electron transfer. Recent studies suggest that a wide variety of CI inhibitors share a common binding domain at or close to the ubiquinone reduction site (Ino et al., 2003). Furthermore, the structural factors required for inhibitory actions have been characterized on the basis of structure-activity relationships (Miyoshi, 1998, Hideto, 1998). Based on molecular docking simulations, in silico models mimicking the binding of chemicals to the pocket of NADH ubiquinone oxidoreductase have been created according to the crystal structure of mitochondrial CI. To investigate the ability of chemicals to bind to the active pocket, around 100 individual docking simulations have been performed. These confirmed the possible site of interaction between the chemical and the pocket of CI. In particular, Miao YJ and coworkers recently investigated the IC50 values of 24 chemicals (annonaceous acetogenins) for inhibition of mitochondrial CI (Miao et al., 2014).

Based on their binding sites, CI inhibitors are classified as follows (Degli Esposti, 1998) (Fig. 2): (i) type A inhibitors are antagonists of fully oxidized ubiquinone binding; (ii) type B inhibitors displace the partially reduced ubisemiquinone intermediate; (iii) type C inhibitors are antagonists of the fully reduced ubiquinol product. The affinity of the different types of CI inhibitors to their diverse CI binding sites is described in the paragraph Evidence for Chemical Initiation of this Molecular Initiating Event (see below) in the context of a specific type of inhibitor.

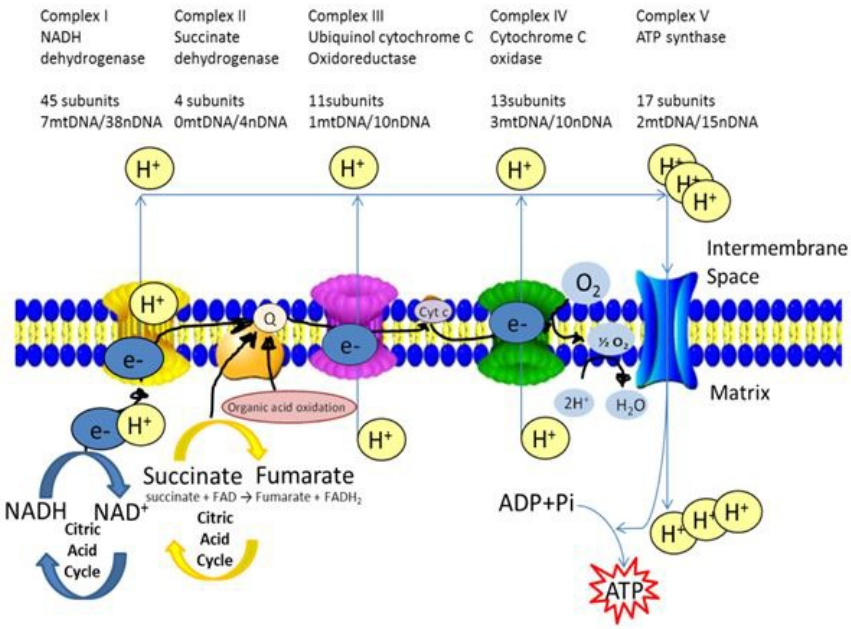

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Fig. 1. The electron transport chain in the mitochondrion. CI (NADH-coenzyme Q reductase or NADH dehydrogenase) accepts electrons from NADH and serves as the link between glycolysis, the citric acid cycle, fatty acid oxidation and the electron transport chain. Complex II also known as succinate-coenzyme Q reductase or succinate dehydrogenase, includes succinate dehydrogenase and serves as a direct link between the citric acid cycle and the electron transport chain. The coenzyme Q reductase or Complex III transfers the electrons from CoQH2 to reduce cytochrome c which is the substrate for Complex IV (cytochrome c reductase). Complex IV transfers the electrons from cytochrome c to reduce molecular oxygen into water. Finally, this gradient is used by the ATP synthase complex (Complex V) to make ATP via oxidative phosphorylation. mtDNA: mitochondrial DNA; nDNA: nuclear DNA.

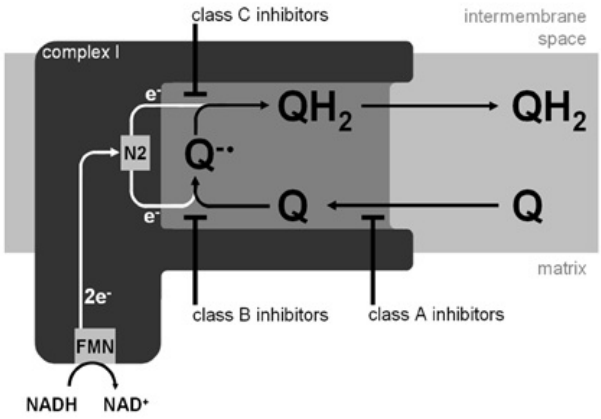

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Fig. 2. Schematic representation of CI and proposed inhibition binding sites by inhibitors of class A, B and C. Nicotinamide adenine dinucleotide (NADH, reduced and NAD, oxidized), flavin mononucleotide (FMN) and Ubiquinone (Q) (taken from Haefeli, 2012).

How it is Measured or Detected

Two different types of approaches have been used. The first is to measure binding as such, and the corresponding assays are described below; the second is to infer binding indirectly from assays that quantify e.g. CI activity and to assume that the activity can only be altered upon binding. The second type of approach is dealt with in the chapter entitled KE1: Inhibition of NADH ubiquinone oxidoreductase (complex I). However, it has to be noted here that indirect assays can lead to wrong conclusions. For instance, some compounds may trigger oxidative stress without actually binding to CI. Such compounds, by triggering the generation of reactive oxygen species (ROS), may damage CI protein components, thus causing a reduction of CI activity. Measurement of binding by quantitative autoradiography To assess binding of an inhibitor at the rotenone binding site of CI in tissues (e.g. in the substantia nigra or in the striatum), the standard approach is to quantify the displacement of a radioactively labelled ligand of this binding site by the toxicant under evaluation. Most commonly, binding of [3H]-labeled dihydrorotenone (DHR) is measured and compared in control tissue and treated tissue. Binding of this rotenone-derivative is detected by autoradiography. Unselective binding is determined by measurement of [3H]-DHR binding in the presence of an excess of unlabeled rotenone. Since a rotenone-derivative is used for the assay, only CI inhibitors that bind to the rotenone-binding site in CI are detected. This was observed for e.g., meperdine, amobarbital, or MPP+. This method allows a spatial resolution of CI expression and the mapping of the binding of a competitive inhibitor on CI.

The method can be used for (a) in vitro measurements and for (b) ex vivo measurements:

a) In vitro measurements. Tissues are embedded in a matrix for cutting by a cryostat. The tissue slices are then mounted onto slides. For the binding experiment, they are incubated with the test compound in the presence of labeled [3H]-DHR. Then the tissue slices are washed and prepared for autoradiographic detection (Greenamyre et al. 1992; Higgins and Greenamyre, 1996). b) Ex vivo measurements. As rotenone can pass the blood brain barrier, the in vitro method was further extended for in vivo labeling of CI in the brains of living animals, and detection of binding after preparation of the tissue from such animals. Animals are exposed to test compounds and [3H]-DHR is applied intraventricularly for 2-6 h before the brain is dissected and arranged for the preparation of tissue slices (Talpade et al. 2000). In untreated animals, this method allows a precise spatial resolution of the expression pattern of CI. In animals with impaired CI activity, either as a result of CI deficiencies, or upon treatment with CI inhibitors, the assay allows an assessment of the degree of CI inhibition.

Complex I Enzyme Activity (Colorimetric)

The analysis of mitochondrial OXPHOS CI enzyme activity can be performed using human, rat, mouse and bovine cell and tissue extracts (abcam: http://www.abcam.com/complex-i-enzyme-activity-microplate-assay-kit-colorimetric-ab109721 (http://www.abcam.com/complex-i-enzyme-activity-microplate-assay-kit-colorimetric-ab109721)). Capture antibodies specific for CI subunits are pre-coated in the microplate wells. Samples are added to the microplate wells which have been pre-coated with a specific capture antibody. After the target has been immobilized in the well, CI activity is determined by following the oxidation of NADH to NAD+ and the simultaneous reduction of a dye which leads to increased absorbance at OD=450 nm. By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables, an accurate measurement of the enzyme's functional state can be evaluated.

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Key Events

Title	Short name
Inhibition, NADH-ubiquinone oxidoreductase (complex I) (https://aopwiki.org/events/887)	Inhibition, NADH-ubiquinone oxidoreductase (complex I)
N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)	N/A, Mitochondrial dysfunction 1
impaired, Proteostasis (https://aopwiki.org/events/889)	impaired, Proteostasis
N/A, Neuroinflammation (https://aopwiki.org/events/188)	N/A, Neuroinflammation
Degeneration of dopaminergic neurons of the nigrostriatal pathway (https://aopwiki.org/events/890)	Degeneration of dopaminergic neurons of the nigrostriatal pathway

887: Inhibition, NADH-ubiquinone oxidoreductase (complex I) (https://aopwiki.org/events/887)

Short Name: Inhibition, NADH-ubiquinone oxidoreductase (complex I)

AOPs Including This Key Event

AOP ID and Name	Event Type
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent

Stressors

Name
1',2'-dihydrorotenone

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus sp.	Rattus sp.	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10118)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

The CI is well-conserved across species from lower organisms to mammals. The central subunits of CI harboring the bioenergetic core functions are conserved from bacteria to humans. CI from bacteria and from mitochondria of Yarrowia lipolytica, a yeast genetic model for the study of eukaryotic CI (Kerscher et al., 2002) was analyzed by x-ray crystallography (Zickermann et al., 2015, Hofhaus et al., 1991; Baradaran et al., 2013). The CI of the mitochondria of eukaryotes and in the plasma membranes of purple photosynthetic bacteria are closely related to respiratory bacteria and the close homology of sequences, function, and prosthetic groups shows a common ancestry (Friedrich et al., 1995).

How this Key Event Works

Under physiological conditions complex I (CI) couples the oxidation of NADH to NAD+ by reducing flavin mononucleotide (FMN) to FMNH2. FMNH2 is then oxidized through a semiquinone intermediate. Each electron moves from the FMNH2 to Fe-S clusters, and from the Fe-S clusters to ubiquinone (Q). Transfer of the first electron results in the formation of the free-radical (semiquinone) form of Q, and transfer of the second electron reduces the semiquinone form to the ubiquinol form (CoQH2). Altogether, four protons are translocated from the mitochondrial matrix to the inter-membrane space for each molecule of NADH oxidized at CI. This leads to the establishment of the electrochemical potential difference (proton-motive force) that may be used to produce ATP (Garrett and Grisham, 2010). Binding of an inhibitor attenuates or completely blocks the activity of CI, i.e. the oxidation of NADH is impaired and protons are not moved. This causes two major consequences: first, electrons are channelled toward oxygen instead Q. This impairs normal oxygen reduction into water at complex IV and leads to the formation of the ROS superoxide at other sites of the respiratory chain. Superoxide may cause damage of proteins, lipid and DNA of the cell, or damage components of the mitochondria after transformation into e.g. hydrogen peroxide. These processes result in mitochondrial dysfunction (Voet and Voet., 2008). The second consequence is the increase of the NADH/NAD+ ratio in mitochondria. This affects the function of key dehydrogenase enzymes in the citric acid cycle and can lead to its block, resulting in an inhibition of mitochondrial ATP production and mitochondrial respiration. The functional consequences of CI inhibition have been titrated in a time- and dose-dependent manner (Barrientos and Moraes, 1999), with mitochondrial dysfunction measured by a range of different assays (Barrientos and Moraes, 1999; Greenamyre et al., 2001). These included quantification of ROS derived from mitochondria, and of cellular respiration (see KE2: Mitochondrial dysfunction).

How it is Measured or Detected

As CI has an enzymatic function as such, but also contributes to the overall function of oxidative phosphorylation, there are two fundamental approaches to assess CI inhibition. The first approach measures the enzymatic activity of the complex itself; the second one assesses the overall activity of oxidative phosphorylation of entire mitochondria, and indirectly infers from this a potential dysfunction of CI.

I. Direct detection of complex I activity This type of assay is always performed in homogenates of cells or tissues, and requires at least a partial purification of mitochondria or respiratory chain components. In order to focus on CI activity, the activities of Complexes III (e.g. antimycin A) and complex IV (e.g. cyanide) need to be blocked by pharmacological inhibitors in these setups.

1. Forward Electron Transfer Submitochondrial particles or intact isolated mitochondria are incubated with NADH as electron donor and with an electron acceptor to measure the flow of electrons from NADH, through CI to the acceptor. As readout, either the consumption of NADH, or the reduction of the electron acceptor is followed photometrically or fluorometrically (Lenaz et al. 2004; Spinazzi et al. 2012; Long et al. 2009; Kirby et al. 2007). The physiological electron acceptor of CI is Coenzyme Q10 (CoQ10). Due to its hydrophobicity, it is not suitable

for use in an experimental in vitro setup. Short-chain analogs of CoQ10, such as CoQ1 or decylubiquinone (DB) with a 10 carbon-atom linear saturated side chain are hence applied as alternatives. With these non-physiological electron acceptors, it is important to consider that the activity of CI can easily be underestimated. As water-soluble electron acceptors, either ferricyanide or 2,6-dichlorophenolidophenol (DCIP) are used. However the reduction of such compounds is not strictly coupled to the transduction of energy. To identify the portion of rotenone-inhibitable CI activity, all samples investigated are assayed in parallel following treatment with rotenone. In contrast to the autoradiography assays, direct CI activity detection allows the identification also of CI inhibitors that bind to sites of CI different from the rotenone binding site.

2. Reverse Electron Transfer An alternative setup for the direct measurement of CI activity with minimal interference by the activities of complex III and complex IV make use of the observation of a general reversibility of oxidative phosphorylation and electron flow across the mitochondrial respiratory chain (Ernster et al. 1967). With this method, electrons enter the respiratory chain via complex II. Based on the reverse flux, this method allows the complete circumvention of complexes III and IV. As electron donor, succinate is applied, together with NAD+ as electron acceptor. Formation of NADH from NAD+ can be determined photometrically. The succinate-linked NAD+ reduction can be performed either with intact isolated mitochondria or with submitochondrial particles. For the direct assessment of CI activity, submitochondrial particles are used. For assays with intact mitochondria, the succinate-linked reduction of NAD+ is performed in the presence of ATP as energy source. Potassium cyanide (KCN) is added for inhibition of forward electron transport towards complex IV.

3. Complex I activity dipstick assay To assess CI activity and its inhibition in cell or tissue homogenates without interference by other components of the respiratory chain, CI-selective antibodies attached to a matrix (e.g. multiwell plates) are used (Willis et al., 2009). Homogenized tissue can directly be added for capturing of CI, the unbound supernatant is washed away and leaves a complex of the antibody and mitochondrial CI. For activity determination, NADH as electron donor and nitroblue tetrazolium (NBT) as acceptor are added. Reduced NBT forms a colored precipitate, its signal intensity is proportional to the amount of CI bound to the antibody. CI inhibitors can directly be added for an assessment of their inhibitory potential. This method, when applied in e.g. 96-well or 384-well plates, allows screening of large sets of potential CI inhibitors without any interference by other elements of the mitochondrial respiratory chain.

II. Indirect measurements of complex I activity. Such assays mostly require / allow the use of live cells.

1. Oxygen consumption Electrons, fed into the mitochondrial respiratory chain either by CI or complex II, ultimately reduce molecular oxygen to water at complex IV. In a closed system, this consumption of oxygen leads to a drop of the overall O2 concentration, and this can serve as parameter for mitochondrial respiratory activity. Measurements are traditionally done with a Clark electrode, or with more sophisticated optical methods. At the cathode of a Clark electrode, oxygen is electrolytically reduced, which initiates a current in the electrode, causing a potential difference that is ultimately recorded. Clark electrodes however have the disadvantage that oxygen is consumed. Furthermore, interferences with nitrogen oxides, ozone, or chlorine are observed (Stetter et al., 2008). To circumvent these limitations, optical sensors have been developed that have the advantage that no oxygen is consumed, combined with a high accuracy and reversibility. Optical oxygen sensors work according to the principle of dynamic fluorescence quenching. The response of the respective fluorescence dye is proportional to the amount of oxygen in the sample investigated (Wang and Wolfbeis, 2014). In a model of isolated mitochondria in the absence of complex II substrates, oxygen consumption can serve as surrogate readout for the assessment of the degree of CI inhibition. It is however essential to realize that also complex III and complex IV activities are involved and their inhibition also results in a decline in O2 consumption. In addition to that, CI inhibitors can lead to a one-electron reduction of molecular oxygen at the site of CI to yield superoxide. The amount of superoxide formed hence contributes to the consumption of oxygen, but this must not be interpreted as oxygen consumption as a result of controlled and coupled electron flux through the complexes of the mitochondrial respiratory chain. A modern convenient method to measure oxygen consumption is provided by the Seahorse technology of extracellular flux (XF) analysis, in which cells are kept in a very small volume, so that changes of oxygen levels can be detected very sensitively by an oxygen sensor. To allow manipulation of the mitochondria in cells, the cell membrane can be permeabilized with saponin (SAP), digitonin (DIG) or recombinant perfringolysin O (rPFO) (XF-plasma membrane permeabilizer (PMP) reagent), to allow addition of specific substrates to measure activity of different respiratory chain complexes, including CI. (Salabei et al., 2014).

2. Intracellular ATP levels Intracellular ATP levels originate both from mitochondria and from glycolysis. If glycolytic ATP production is impaired or inhibited, the cellular production of ATP is a measure of mitochondrial function. If it is assumed that the ATP consumption remains constant, then the steady state ATP levels can serve as indirect readout for mitochondrial activity, and the latter depends on the functioning of CI. Inhibitors of CI reduce cellular ATP levels, but it has to be remembered that intracellular ATP levels are also affected by inhibitors of other parts of the respiratory chain, of the citric acid cycle or of the transport of energy substrates. For a proper interpretation of assay results, it has to be ascertained in each particular test system, that ATP production from other sources is excluded and that the cellular ATP consumption remains constant. ATP levels can be easily measured from lysates of in vitro cell cultures or from tissues by a luminometric luciferase/luciferin assay. The amount of light emitted is proportional to the amount of ATP in the sample (Nguyen et al. 1988) (Leist, 1997).

3. Other approaches As mitochondrial activity is coupled to many cellular functions, there is a multitude of other

indirect assays that are sensitive to inhibitors of CI. Some of these tests may indeed be very sensitive, while they have a low specificity. Thus, their application requires usually a good control of the experimental system and care with the interpretation of the data. One exemplary approach is the measurement of NADH/NAD+ ratios in mitochondria by imaging methods. This provides resolution on the level of individual mitochondria within a living cell (van Vliet et al., 2014)

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177: N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)

Short Name: N/A, Mitochondrial dysfunction 1

AOPs Including This Key Event

AOP ID and Name	Event Type
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
77: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1 (https://aopwiki.org/aops/77)	KeyEvent
78: Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent
79: Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure (https://aopwiki.org/aops/79)	KeyEvent
80: Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure (https://aopwiki.org/aops/80)	KeyEvent
87: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 2 (https://aopwiki.org/aops/87)	KeyEvent
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent
144: Lysosomal damage leading to liver inflammation (https://aopwiki.org/aops/144)	KeyEvent
178: Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure (https://aopwiki.org/aops/178)	KeyEvent
200: Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Mitochondrial dysfunction is a universal event occurring in cells of any species (Farooqui and Farooqui, 2012). Many invertebrate species (drosophila, C, elegans) are considered as potential models to study mitochondrial function. New data on marine invertebrates, such as molluscs and crustaceans and non-Drosophila species, are emerging (Martinez-Cruz et al., 2012). Mitochondrial dysfunction can be measured in animal models used for toxicity testing (Winklhofer and Haass, 2010; Waerzeggers et al 2010) as well as in humans (Winklhofer and Haass, 2010).

How this Key Event Works

Mitochondrial dysfunction is a consequence of inhibition of the respiratory chain leading to oxidative stress.

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O2-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and peroxiredoxins in various cell organelles (reviewed in Lin and Beal, 2006). It is worth mentioning that, as in the case of ROS generation, antioxidant defences are also closely related to the redox and energetic status of mitochondria. If mitochondria are structurally and functionally healthy, an antioxidant defence mechanism balances ROS generation, and there is not much available ROS production. However, in case of mitochondrial damage, the antioxidant defence capacity drops and ROS generation takes over. Once this happens, a vicious cycle starts and ROS can further damage mitochondria, leading to more free-radical generation and further loss of antioxidant capacity. During mitochondrial dysfunction the availability of ATP also decreases, which is considered necessary for repair mechanisms after ROS generation.

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM)are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca2+ signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca2+ at the MAM and increased Ca2+ uptake into the mitochondrial matrix, which induces Ca2+-dependent mitochondrial outer membrane permeabilization and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca2+, increased mitochondrial uptake of Ca2+, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca2+ flux which induce ER stress. Increased Ca2+ flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition pore (MPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca2+ signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca2+ homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013).). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca2+ uptake, causing Ca2+ overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c (Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

Mitochondrial dysfunction can be detected using isolated mitochondria, intact cells or cells in culture as well as in vivo studies. Such assessment can be performed with a large range of methods (revised by Brand and Nicholls, 2011) for which some important examples are given. All approaches to assess mitochondrial dysfunction fall into two main categories: the first assesses the consequences of a loss-of-function, i.e. impaired functioning of the respiratory chain and processes linked to it. Some assay to assess this have been described for KE1, with the limitation that they are not specific for complex I. In the context of overall mitochondrial dysfunction, the same assays provide useful information, when performed under slightly different

assay conditions (e.g. without addition of complex III and IV inhibitors). The second approach assesses a ‘non-desirable gain-of-function’, i.e. processes that are usually only present to a very small degree in healthy cells, and that are triggered in a cell, in which mitochondria fail.

I. Mitochondrial dysfunction assays assessing a loss-of function.

1. Cellular oxygen consumption

See KE1 for details of oxygen consumption assays. The oxygen consumption parameter can be combined with other endpoints to derive more specific information on the efficacy of mitochondrial function. One approach measures the ADP-to-O ratio (the number of ADP molecules phosphorylated per oxygen atom reduced (Hinkle, 1995 and Hafner et al., 1990). The related P/O ratio is calculated from the amount of ADP added, divided by the amount of O consumed while phosphorylating the added ADP (Ciapaite et al., 2005; Diepart et al., 2010; Hynes et al., 2006; James et al., 1995; von Heimburg et al., 2005).

2. Mitochondrial membrane potential (Δψm)

The mitochondrial membrane potential (Δψm) is the electric potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. The classical, and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodamine, methyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos et al., 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

3. Enzymatic activity of the electron transport system (ETS)

Determination of ETS activity can be determined following Owens and King's assay (1975). The technique is based on a cell-free homogenate that is incubated with NADH to saturate the mitochondrial ETS and an artificial electron acceptor [l - (4 -iodophenyl) -3 - (4 -nitrophenyl) -5-phenylte trazolium chloride (INT)] to register the electron transmission rate. The oxygen consumption rate is calculated from the molar production rate of INT-formazan which is determined spectrophotometrically (Cammen et al., 1990).

4. ATP content

For the evaluation of ATP levels, various commercially-available ATP assay kits are offered (e.g. Sigma, http://www.abcam.com/atp-assay-kit-colorimetricfluorometric-ab83355.html (http://www.abcam.com/atp-assay-kit-colorimetricfluorometric-ab83355.html)), based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., (2005).

II. Mitochondrial dysfunction assays assessing a gain-of function.

1. Mitochondrial permeability transition pore opening (PTP)

The opening of the PTP is associated with a permeabilization of mitochondrial membranes, so that different compounds and cellular constituents can change intracellular localization. This can be measured by assessment of the translocation of cytochrome c, adenylate kinase or AIF from mitochondria to the cytosol or nucleus. The translocation can be assessed biochemically in cell fractions, by imaging approaches in fixed cells or tissues or by life-cell imaging of GFP fusion proteins (Single 1998; Modjtahedi 2006). An alternative approach is to measure the accessibility of cobalt to the mitochondrial matrix in a calcein fluorescence quenching assay in live permeabilized cells (Petronilli et al., 1999).

2. mtDNA damage as a biomarker of mitochondrial dysfunction

Various quantitative polymerase chain reaction (QPCR)-based assays have been developed to detect changes of DNA structure and sequence in the mitochondrial genome. mtDNA damage can be detected in blood after low-level rotenone exposure, and the damage persists even after CI activity has returned to normal. With a more sustained rotenone exposure, mtDNA damage is also detected in skeletal muscle. These data support the idea that mtDNA damage in peripheral tissues in the rotenone model may provide a biomarker of past or ongoing mitochondrial toxin exposure (Sanders et al., 2014a and 2014b).

3. Generation of ROS and resultant oxidative stress

a. general approach Electrons from the mitochondrial ETS may be transferred ‘erroneously’ to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent

reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes,etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

b. Measurement of the cellular glutathione (GSH) status GSH is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase (GSSH + NADPH + H+ à 2 GSH + NADP+). The ratio of GSH/GSSG is therefore a good indicator for the cellular NADH+/NADPH ratio (i.e. the redox potential).. GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically with DTNB (Ellman's reagent). As excess GSSG is rapidly exported from most cells to maintain a constant GSH/GSSG ratio, a reduction of total glutathione (GSH/GSSG) is often a good surrogate measure for oxidative stress.

c. Quantification of lipid peroxidation Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde generated from the decomposition of cellular membrane lipid under oxidative stress (Pryor et al., 1976). This method is quite sensitive, but not highly specific.. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be more specific for lipid peroxidation. A number of commercial ELISA kits have been developed for IsoPs, but interfering agents in samples requires partial purification before analysis. Alternatively, GC/MS may be used, as robust (specific) and sensitive method.

d. Detection of superoxide production Generation of superoxide by inhibition of complex I and the methods for its detection are described by Grivennikova and Vinogradov (2014). A range of different methods is also described by BioTek (http://www.biotek.com/resources/articles/reactive-oxygen-species.html (http://www.biotek.com/resources/articles/reactive-oxygen-species.html)). The reduction of ferricytochrome c to ferrocytochrome c may be used to assess the rate of superoxide formation (McCord, 1968). Like in other superoxide assays, specificity can only be obtained by measurements in teh absence and presence of superoxide dismutase. Chemiluminescent reactions have been used for their increased sensitivity. The most widely used chemiluminescent substrate is lucigenin. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical, and they become membrane impermeable after oxidation (trapping at sit of formation). The best characterized of these probes are Hydro-Cy3 and Hydro-Cy5. generation of superoxide in mitochondria can be visualized using fluorescence microscopy with MitoSOX™ Red reagent (Life Technologies). MitoSOX™ Red reagent is a cationic derivative of dihydroethidium that permeates live cells and accumulates in mitochondria.

e. Detection of hydrogen peroxide (H2O2) production There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997: Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H2O2 form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up mitochondrial dysfunction can be measured by: • ROS production: superoxide (O2-), and hydroxyl radicals (OH–) • Nitrosative radical formation such as ONOO– or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (MPTP) • ATP synthesis • Increase in mitochondrial Ca2+ • Cytochrome c release • AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al, 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation (formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ([Ca2+]i): Cells are loaded with 4 µM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion)(Promega assay, TB369; Radkowsky et al., 1986 • Neuronal nitric oxide synthase (nNOS) activation that is Ca2+-dependent All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995). See also KE Oxidative Stress, Increase (https://aopwiki.org/wiki/index.php/Event:209)

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889: impaired, Proteostasis (https://aopwiki.org/events/889)

Short Name: impaired, Proteostasis

AOPs Including This Key Event

AOP ID and Name	Event Type
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent

Stressors

Name
1',2'-dihydrorotenone

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

The ubiquitin proteasome system is highly conserved in eukaryotes, from yeast to human. Ubiquitin is a small (8.5 kDa) regulatory protein that has been found in almost all tissues of eukaryotic organisms. For instance, drosophila has been used as PD model to study the role of ubiquitin in α-synuclein induced-toxicity (Lee et al., 2009). Human and yeast ubiquitin share 96% sequence identity. Neither ubiquitin nor the ubiquitination machinery are known to exist in prokaryotes. Autophagy is ubiquitous in eukaryotic cells and is the major mechanism involved in the clearance of oxidatively or otherwise damaged/worn-out macromolecules and organelles (Esteves et al., 2011). Due to the high degree of conservation, most of the knowledge on autophagy proteins in vertebrates is derived from studies in yeast (Klionsky et al., 2007). Autophagy is seen in all eukaryotic systems, including fungi, plants, slime mold, nematodes, fruit flies and insects, rodents (i.e., laboratory mice and rats), and humans. It is a fundamental and phylogenetically conserved self-degradation process that is characterized by the formation of double-layered vesicles (autophagosomes) around intracellular cargo for delivery to lysosomes and proteolytic degradation.

How this Key Event Works

The concept of proteostasis refers to the homeostasis of proteins in space and time, i.e. the correct balance between protein synthesis, modification, transport and degradation. Disturbance of proteostasis results in pathological changes either by loss of function events (lack of a pivotal protein/protein function) or by a gain of undesired functions (aggregation of a protein leading to the formation of inclusions and new structures in cells and disturbing turnover of many unrelated proteins).

Proteostasis regulation is the main defence mechanism against toxic proteins, whose accumulation could greatly compromise normal cellular function and viability. Therefore, the chaperone and degradation systems assuring the removal of misfolded and aggregated proteins, as well as damaged, dysfunctional cellular organelles (e.g., defective mitochondria) play a key role in cellular homeostasis (Lee et al., 2012). The two major degradation systems are the ubiquitin–proteasome system (UPS) and the autophagy–lysosome pathway (ALP) (Korolchuk et al., 2010; Kroemer et al., 2010; Ravikumar et al., 2010). The UPS works through the attachment of multiple ubiquitin molecules to a protein substrate, followed by the subsequent degradation of the tagged polyubiquitinated protein by the proteasome (Ciechanover, 1998; Ciechanover and Brundin, 2003). A compromised function of the UPS leads to the accumulation of ubiquitylated proteins, such as α-synuclein, (Ii et al. 1997; Spillantini et al. 1997; Sulzer and Zecca 2000). The accumulation of polyubiquitinated proteins, as a consequence of a dysfunctional proteasome activity, is observed in some pathologies, and experimental inhibition of the proteasome has been shown to trigger parkinsonian neurodegeneration (McNaught and Jenner 2001; Hardy et al., 2001).

ALP involves the engulfment of cytoplasmic materials into autophagosomes, which are degraded by lysosomal enzymes after fusion of autophagosomes with lysosomes (Kuma et al., 2004) or direct import of proteins into lysosomes (Cuervo, 2004; Mizushima et al., 2008). Autophagy also plays an essential role for the removal of damaged organelles, such as mitochondria. Both, excessive autophagy or reduced autophagic flux can compromise cell survival (Rothermel and Hill, 2007), and several genetic forms of PD are linked to the autophagy-related genes Pink1, Parkin or Uchl1. Autophagy enables cell survival during mitochondrial stress by clearing the damaged organelles (Lee et al., 2012).

One of the main aggregated proteins found to accumulate in nigrostriatal cells during Parkinson's disease is α-synuclein. Aggregation of α-synuclein can obstruct normal cellular transport, leading to impaired intracellular trafficking and/or trapping of cellular organelles in inappropriate locations, this resulting in synaptic and cell dysfunctions (Bartels et al., 2011) (Bellucci A., et al., 2012; Cookson MR., 2005; Games D., et al., 2013; Hunn BH., et al., 2015). Importantly, accumulation of α-synuclein affects mitochondrial trafficking. The polarity and correct function of different types of cells depend on an efficient transport of mitochondria to areas of high energy consumption (Sheng, 2014). Therefore, the correct distribution of mitochondria to various parts of a cell is essential to preserve cell function (Schwarz, 2013; Zhu et al., 2012).

How it is Measured or Detected

1. Evaluation of UPS function General turnover assays Quantitative evaluation can be based on the detection of increased ubiquitin or ubiquinated proteins, as well as proteasomal subunits, either by immunocyto/histochemistry or by western blotting (Rideout et al., 2001; Ortega and Lucas, 2014). UPS activity can be continuously monitored by quantitating (by mean of flow cytometry or microscopy) the level of e.g. EGFP-degron fusion proteins that are selectively degraded by the proteasome (Bence et al., 2001).

Proteasome activity assay Various fluorogenic substrates (e.g., Suc-Leu-Leu-Val-Tyr-AMC for the chymotrypsin-like activity) can be used for the determination of proteasomal activity in in vivo or in vitro applications. These substrates may be applied to tissue or cell homogenates, but specific measurements require partial purification of the proteasome (Kisselev and Goldberg, 2005).

Detection of α-synuclein (AS) aggregates The most common methods to detect AS aggregates use immunostaining for AS (in cells or in tissues). In cell culture, AS may also be epitope-tagged or coupled to GFP to allow an indirect detection. The detection of small, not microscopically-visible AS aggregates is indicative of protease-resistance. Tissue slices may be exposed to proteases before immunostaining for AS. Alternatively, small or large aggregates may be biochemically enriched by differential centrifugation and proteolytic treatment, and then analyzed, e.g., by western blot, mass spectrometry or ELISA-like immunoquantification.

2. Evaluation of ALP function Quantification of lysosomes or autophagosomes Disturbances of ALP often result in counter-regulations that can be visualized by staining of lysosomes or parts of the autophagy system. Several weakly basic dyes can be used to stain acidic organelles (lysosomes) in live cells. For example, the dye LysoTracker Red stains lysosomes and can be used to monitor autophagy (Klionsky et al., 2007; Klionsky et al., 2008). The autofluorescent drug monodansylcadaverine (MDC) has also been used as autophago-lysosome marker (Munafó and Colombo, 2002). A convenient way to stain lysosomes in tissue or fixed cells is the use of antibodies against the Lysosomal-Associated Membrane Protein 1 (LAMP-1) (Rajapakshe et al., 2015) or against cathepsins (Foghsgaard et al., 2001). For qualitative or semiquantitative estimates of lysosomes and related organelles, transmission electron microscopy has been frequently used (Barth et al., 2010).

Monitoring of autophagy-related molecules The amount and the localization of autophagy-related proteins can change during disturbance of the ALP. Especially in cell culture, but also in transgenic mice, various techniques have been used to monitor autophagy by mean of fluorescence-tags or other substrates, e.g., ATG, autophagy-

related protein or autophagy substrates, to monitor their fate in cells and thus provide information on disturbed ALP, or the over-expression of GFP–LC3, in which GFP (green fluorescent protein) is expressed as a fusion protein at the amino terminus of LC3 (microtubule-associated protein 1A/1B-light chain 3), which is the a mammalian homologue of S. cerevisiae ATG8 (Kadowaki and Karim, 2009).

Monitoring autophagic flux The lysosomal degradation of the autophagic cargo constitutes the autophagic flux, which can be measured by assessing the rate of turnover of long-lived proteins that are normally turned over by autophagy (Bauvy et al., 2009) This is performed by labelling intracellular proteins with either [14C]-leucine or [14C]-valine, followed by a long culture period in standard medium. The release of radioactive leucin or valin into the culture medium corresponds to the protein degradation rate in cells, and it may be measured by liquid scintillation counting.

Monitoring the conversion of LC3-I to LC3-II The progression of autophagy (autophagic flux) can be studied by the conversion of LC3-I into LC3-II (i.e. a post-translational modification specific for autophagy) by mean of Western blot analysis. The amount of LC3-II correlates with the number of autophagosomes. Conversion of LC3 can be used to examine autophagic activity in the presence or absence of lysosomal activity (Klionsky et al., 2007; Klionsky et al., 2008). The technology can also be used in vivo, e.g. by the use of transgenic mice that overexpress GFP–LC3 (Kuma et al., 2004).

3. Evaluation of intracellular transport of mitochondria and other organelles A range of technologies has been used to visualize mitochondrial dynamics in live cells (Jakobs, 2006; Grafstein and Forman, 1980). They usually employ a combination of mitochondrial labelling with fluorescent dyes (e.g. DiOC6 (3, 3′-Dihexyloxacarbocyanine iodide), JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′ tetraethylbenzimida-zolylcarbo-cyanine iodide), MitoTracker, MitoFluor probes, etc.), followed by video- or confocal microscopy for live cell imaging (Schwarz, 2013; Pool et al., 2006). Most frequently, mitochondrial mobility is observed along neurites, and measurable endpoints may be mitochondrial speed and direction with regard to the cell soma (Schildknecht et al. 2013). Additionally, also mitochondrial fusion and fission have been monitored by such methods (Exner et al., 2012). The transport of other organelles along neurites may be monitored using similar methods, and the microtubule structures that serve as transport scaffold may be co-stained.

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188: N/A, Neuroinflammation (https://aopwiki.org/events/188)

Short Name: N/A, Neuroinflammation

AOPs Including This Key Event

AOP ID and Name	Event Type
17: Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration (https://aopwiki.org/aops/17)	KeyEvent
12: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging (https://aopwiki.org/aops/12)	KeyEvent
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent

Biological Organization

Level of Biological Organization
Tissue

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Neuroinflammation is observed in humans, monkey, rat, mouse and zebrafish associated to neurodegeneration or following toxicant exposure. Some references (not exhaustive list) are given below for illustration:

In humans: Vennetti et al., 2006; In monkey (Macaca fascicularis): Charleston et al., 1994, 1996; In rat: Little et al., 2012; Eskes et al., 2002; In mouse: Liu et al., 2012; In zebrafish: Xu et al., 2014

How this Key Event Works

Neuroinflammation or brain inflammation differs from peripheral inflammation in that the vascular response and the role of peripheral bone marrow-derived cells are less conspicuous. The most easily detectable feature of neuroinflammation is the activation of microglial cells and astrocytes.It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of these glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al, 2007; Streit et al, 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines or inflammogens (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signalling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001), as well as in the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signalling and other cellular functions (such as phagocytosis)

(Streit et al., 1999; Nakajima and Kohsaka, 2004). Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006 ; Monnet-Tschudi et al, 2007; Aguzzi et al., 2013 ; Glass et al., 2010). Under normal physiological conditions, microglial cells scan the nervous system for neural integrity (Nimmerjahn et al, 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defence), and can subsequently induceastrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al, 2009; Maresz et al, 2008; Mosser & Edwards, 2008; Perego et al; Ponomarev et al, 2005): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al, 2008; Perego et al, 2011; Ponomarev et al, 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006).

How it is Measured or Detected

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation: 1. Microglial activati*on can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells). A specific microglial marker, used across different species, is CD11b. Alternatively, various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains.* 1. The most frequently used astrocyte marker is GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and good clinically-validated antibodies are available for immunocytochemical detection. In neuroinflamatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin of the S100beta protein have been used for staining of astrocytes (Struzynska et al., 2007). 2. All immunocytochemical methods can also be applied to cell culture models. 3. In patients, microglial accumulation can be monitored by PET imaging, using [11C]-PK 11195 as microglial marker (Banati et al., 2002). 4. Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of activation markers. This can for instance be done by PCR quantification of inflammatory factors, of by measurement of the respective mediators, e.g. by ELISA-related immuno-quantification. Such markers include: • Pro- and anti-inflammatory cytokine expression (IL-1β; TNF-α, Il-6, IL-4) ; or expression of immunostimmulatory proteins (e.g. MHC-II) • Itgam, CD86 expression as markers of M1 microglial phenotype • Arg1, MRC1, as markers of M2 microglial phenotype (for description of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014)

Regulatory example using the KE:

Measurement of glial fibrillary acidic protein (GFAP) in brain tissue, whose increase is a marker of astrocyte reactivity, is required by the US EPA in rodent toxicity studies for fuel additives (40 CFR 79.67), but is optional for other toxicant evaluations..

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890: Degeneration of dopaminergic neurons of the nigrostriatal pathway (https://aopwiki.org/events/890)

Short Name: Degeneration of dopaminergic neurons of the nigrostriatal pathway

AOPs Including This Key Event

AOP ID and Name	Event Type
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Parkinson's disease (PD), one of the best characterized parkinsonian disorder, is a progressive age-related human neurodegenerative disease with a multi-factorial pathogenesis implicating various genetic and environmental factors and is more prevalent in males (Fujita et al. 2014). However, the anatomy and function of the nigrostriatal pathway is conserved across mammalian species (Barron et al. 2010).

Pathological changes, similar to the one described in human PD, have been reproduced with chemicals such as rotenone and MPTP. These chemicals have been tested successfully in multiple mammalian species, including primates, rats and mice. The mouse C57BL/6 strain is the most frequently used strain in the reported experiments. A difference in vulnerability was observed, particularly for rats, depending on the strain and route of administration, possibly indicating the relevance of genetic factors in the development of this pathology. The Lewis strain gives more consistency in terms of sensitivity when compared to the Sprague Dawley. In addition to rodents, the pesticide rotenone has been also studied in Caenorhabditis elegans (C.elegans), Drosophila, Zebrafish and Lymnaea Stagnalis (L.stagnalis) (Johnson et al., 2015).

How this Key Event Works

Degeneration of dopaminergic neurons (DA neurons) within the Substantia Nigra pars compacta (SNpc) i.e. the nigrostriatal pathway, paralleled by the formation of cytoplasmic fibrillar inclusions called Lewy bodies (LB), is regarded as a key event in Parkinson's disease (PD) and is, in a quantitative manner, directly linked to the occurrence of clinical signs indicative of PD, i..e impaired motor behavior (Shulman et al. 2011; Jellinger et al.

2009, Dickinson 2012, Dauer et al. 2003). The severity of the clinical signs correlates with the degree of nigral cell loss, and the reduced level of dopamine in the striatum. It is estimated that at the onset of clinical signs, 60% of SNpc neurons are lost, corresponding to an 80% depletion of striatal dopamine (Jellinger et al. 2009). PD is clinically and pathologically defined as a progressive disorder: There is a temporally progress, according to a specific pattern, from the brain stem to the nigrostriatal areas and to cortical locations (Braak et al. 2004 and 2009) and there is a temporal increase in the occurrence of LB, of dopamine depletion in the striatum and of loss of DA neurons in the SNpc (Shulman et al. 2012). Indeed, in patients with PD there is a more evident loss of dopamine in striatum compared to SNpc, indicating that striatal dopaminergic nerve terminals are the primary target of the degenerative process in the nigrostriatal pathway and that neuronal loss in SNpc would result as a final outcome (Hornykiewicz et al.1966; Dauer et al. 2003; Bernhaimer et al. 1973; Pavese. et al. 2009). Studies in PD patients and experimental models are also suggesting that progression from striatal terminal to loss of DA neurons occurs through a “dying back” axonopathy pathology and that axonal dysfuction may be an important hallmark in PD (Orimo et al. 2005; Raff et. al. 2002; Kim-Han et al. 2011; O’Malley 2010).

In human brain, the classical Lewy body (LB) is characterized at light microscopy by eosinophilic, spherical, intra-cytoplasmatic inclusion and it stains for α-synuclein and ubiqutin proteins which form the ultrastructural fibrillar core of LB visible at transmission electron microscopy. On autopsy, from individuals affected by PD, accumulation of aggregates positive for α-synuclein protein are also observed within neuronal processes, called Lewy neurites (LN), as well as by neurons showing a more diffuse or granular peri-nuclear pattern (Dickson 2012). Because dopaminergic cells are rich in melanin, their loss is detectable by depigmentation of the midbrain at gross pathology examination (Dickson 2012; Shulman et al. 2010). However, it should be noted that, although LB are recognized as characteristic of PD, they are not found in a minority of clinically defined PD cases (Dauer 2003) and they can also be observed in other diseases (Dickson 2012).

The biological function of the nigrostriatal pathway depends on the intactness of its anatomical structure. Preservation of the striatum terminals and of neuronal cell bodies of DA neurons in the SNpc is a prerequisite for the maintenance of the physiological function (Fujita et al. 2014). The nigrostriatal system is anatomically located in the basal ganglia loop which comprises the motor system structures caudate nucleus, putamen, globus pallidum and subsatantia nigra. The caudate nucleus and the putamen are collectively called striatum (David Robinson in: Neurobiology, Springer edition, 1997). The system plays a unique integrative role in the control of movement as part of a system called the “basal ganglia motor loop”. This anatomical loop includes structures in the thalamus, motor and somatosensory cortex and wide regions of surrounding cortex. Neurons of the SN produce dopamine (DA) and project to the striatum. They give dopaminergic excitatory (D1 receptors) and inhibitory (D2 receptors) inputs to striatal interneurons (GABAergic). These control thalamic output to the motor cortex. Degeneration within the SNpc leads to a decreased thalamic activation of the motor cortex. (Shulman et al, 2011).

The dopaminergic cells localized in the SNpc synthesize the transmitter substance dopamine (DA) and make extensive contacts within the caudate and putamen (the striatum). These DA neurons have a complex morphology and high energy demand. They are provided with very long and dense arborisations projecting into the striatum where DA is released. This unique morphological characteristics demand a high level of energy to maintain the activity at the synaptic level, to compensate for the risk of depolarization of the poorly myelinated fibres and to support a long distance axonal transport. This puts a tremendous burden on mitochondrial functions (Pissadaki et al. 2013). SNpc neurons are provided with specific calcium channels, the L-type Cav 1.3 which are intended to regulate the autonomous firing as “pacemaker”. The high demand of calcium buffering arising from this is handled by the endoplasmic reticulum (ER) and by the mitochondria. This is a function specific for SNpc DA neurons, as the dopaminergic neurons belonging to the ventral tegmental area (VTA) are using Na+ channels as a pacemaker. Additional peculiarities of the neurons of the nigrostriatal pathway are the high number of synapses and the higher probability of these neurons to accumulate misfolded proteins, including α-synuclein. Furthermore, the nigrostriatal pathway metabolism of DA is known to induce oxidative and nitrative stress (Fujita et al.2014; Asanuma et al. 2003; Cantuti-Castelvetri et al. 2003; Pissadaki et al. 2013) making DA neurons particularly sensitive to oxidative stress (Lotharius and Brundin, 2002). DA neurons in SNpc also have a relatively low mitochondria mass which may contribute to the vulnerability of these neurons (Liang et al. 2007). In addition, increased levels of iron have been observed in SN of PD patients (Gotz et al. 2004) and the high content of iron in dopamine neurons has been reported to trigger oxidative/nitrosative stress and subsequent neurodegeneration (Ayton and Lei 2014; Benshachar et al. 1991). As a consequence, these neurons are particularly sensitive to various stressors that can contribute to their vulnerability and preferential loss (Fujita et al. 2014).

How it is Measured or Detected

The presence of DA cells in the SNpc and DA terminals in the striatum can be visualized using different phenotypic histological markers. Changes can be captured by measurement of markers specific for dopaminergic neurons such as tyrosine hydroxylase dopamine transporter (DAT) and vesicular monoamine transporter type 2 (VMAT2). Degenerating and/or degenerated neurons can be detected by the silver stains and the Fluoro Jade stains.

• The silver degeneration stain is a method to trace degeneration of axons. By this matter, products from disintegrated cells are visualized (Switzer R., 2000; Betarbet et al. 2000). The mechanism by which the siver degeneration stain labels degenerating neurons is unknown.

• Fluoro Jade stain is a fluorochrome derived from fluorescein used in neuroscience disciplines to label degenerating neurons. It is an alternative technique to traditional methods for labeling degenerating neurons such as silver degeneration staining. Fluoro-Jade may be preferred to other degeneration stains due to the simplicity of staining procedures, which are a common drawbacks of conventional stains. However, the mechanism by which fluoro-jade labels degenerating neurons is unknown (Betarbet et al. 2000, Schmued et al. 1997).

• Detection of TH, the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor for dopamine. Detection of TH can be done either by immunocytochemistry followed by cell counting (quantitative evaluation) or by western blot followed by densitometry analysis (Betarbet et al. 2000, Lee 1987, Fetissov 1999).

• Counting of cells, immunostained for TH, or counting of nuclei by e.g. with Nissel's , DAPI (Kapuscinski, 1995) or Hoechst stain (Latt et al. 1976) should be done following standard morphometric protocols. However, inclusion of stereological cell counts to assess neurodegeneration is representing the most sensitive method to confirm quantitatively specific morphological changes (Dauer 2008, Brooks 1992, Thiruchelvam 2000a and 2000b).

• Quantification of dopaminergic neurons in SNpc: the average number of DA neurons in adult mouse SN is approximately 8.000 to 14.000, depending on strain (Zaborszky and Vadasz 2001). Their distribution is not homogeneous with difference in density between the caudal and rostral part of the SN. The gold standard for counting neurons is then to use an unbiased stereological protocol for cell counting with an optical dissector system (Tieu et al. 2003). This requires a computerized stereology software. The count should include TH+ neurons as well the total count of neurons using a non-specific cell stain (e.g. Nisell's, Fox3).

• Quantification of dopaminergic terminals in the striatum: the density of dopaminergic terminals is not homogeneous in the striatum, increasing from the rostral to the caudal part and representative regions of the striatum should be assessed. This can be done by digitalization of the fibres and quantification by optical density or quantification of the fiber density identifyied by by TH+ immunoreactivity (Tieu et al. 2003; Fernagut et al. 2007). Alternatively, striatal tissue can be isolated for immunobloting of TH or DAT.

• DA transporters (DAT) and vesicular monoamine transporter type 2 (VMAT2) can be visualized and quantified using immunocytochemistry (single cell levels) or western blot followed by densitometry analysis, to quantify the changes in their expression. (Hirata et al. 2007; Fornai et al. 2003; Tong et al. 2011; Ciliax et al. 1995). • DA, DOPAC (DA metabolite) and HVA content in the striatum can be quantified through several methodologies such as capillary electrophoresis, spectrofluorimetry and high performance liquid chromatography (HPLC). The commonly used detectors for chromatography include MS, UV, optical fiber detector, electrochemical detector and fluorescence detector (Zhao et al. 2011, Fornai et al. 2005, Magnusson et al. 1980). • Indentification of LB in standard histological sections stained with haematoxylin and eosin, they are characterized by the presence of pale eosinophilic vacuoles (Betarbet et al. 2000 and 2006; Pappolla et al. 1988; Dale et al. 1992).

• Immuno staining for α-synuclein and ubiquitin to identify and quantify Lewy bodies presence. In vivo, α-synuclein and ubiqutin can be evaluated in the fixed tissue and quantified for fluorescence intensity (Betarbet et al. 2000 and 2006; Forno et al. 1996, Tiller-Borcich 1988; Galloway et al. 199;, Kuzuhara et al. 1988; Kuusisto eyt al. 2003).

• Imaging techniques: 18-fluoro-dopa positron emission tomography (PET) quantification of various dopamine presynaptic markers (e.g. dopamine transporter DAT, vescicular monoamine transporter type 2 VAT2) identified by single photon emission tomography (SPECT). They permit to visualize the loss of nigrostriatal DA neurons in patients(Shapira et al. 2013) .

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Adverse Outcomes

Title	Short name
Parkinsonian motor deficits (https://aopwiki.org/events/896)	Parkinsonian motor deficits

896: Parkinsonian motor deficits (https://aopwiki.org/events/896)

Short Name: Parkinsonian motor deficits

AOPs Including This Key Event

AOP ID and Name	Event Type
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Parkinson's disease (PD) is a progressive age-related human neurodegenerative disease with a multi-factorial pathogenesis implicating various genetic and environmental factors and is more prevalent in males (Fujita et al. 2014). There are no sex and species restriction for the application of this AO; however aged animals showed to be more suscieptible to parkinsonian motor deficits induced by chemical stressors (Rose et al.1993, Irwin et al. 1993, Ovadia et al. 1995)

How this Key Event Works

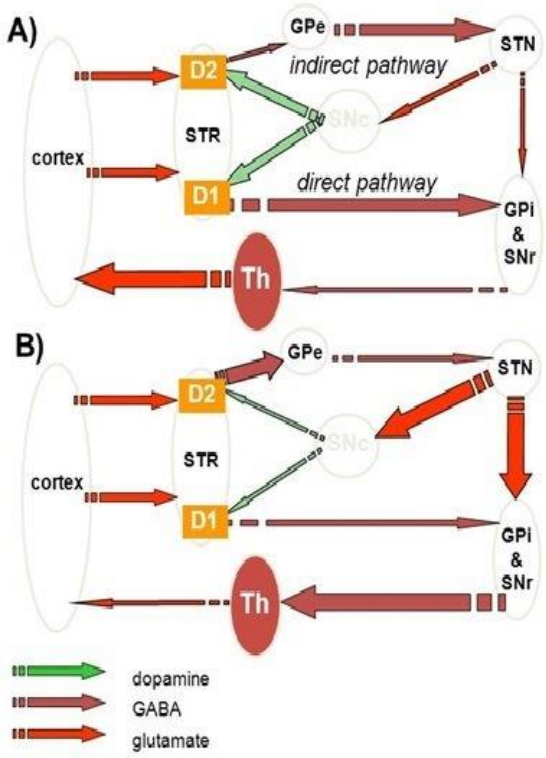
A large number of neurological disorders are characterized by a clinical syndrome with motor symptoms of bradykinesia, tremor, rigidity and postural instability. As these clinical features are common to multiple disorders, the clinical syndrome is referred as "parkinsonism" and when parkinsonism is representing the prevalent part of the syndrome, these are referred as "parkinsonian disorders". Parkinson's Disease (PD) is one of parkinsonian disorders and can have an idiopathic, genetic or toxic (i.e. MPTP induced parkinsonism) cause (Dickson 2012). All these disorders include a deregulation of the extrapyramidal system.

The pyramidal motor system comprises bundles of neurons originating in the motor centers of the cerebral cortex to terminate in the brainstem or in the spinal cord where they are responsible for voluntary control of motor functions (Brooks 1971). The extrapyramidal system, which is the anatomical organization of the AO, is the part of the motor system primarily involved in the control and regulation of involuntary motor control, and in fine tuning (Barnes et al. 1983). Especially the initiation and maintenance of complex movement patterns or of neuronal regulatory pathways involved in postural control of the body are regulated by the nigrostriatal system that is affected in parkinsonian states. The CNS input is modulated by extrapyramidal circuits before the execution of complex motor movements. The modulated information from the basal ganglia is looped back through the thalamus to the cortex, from where final motor signals are sent via the pyramidal system; i.e. the basal ganglia system is not involved in the control of motor neurons and striatal muscles, but it modulates the signals from the cortex to these systems. Thus, an impaired input of dopamine into the striatum leads to an impairment of this modulation loop, and a disturbance of basal ganglia feedback to the thalamus and cortex. This ultimately manifests in key parkinsonian symptoms such as tremor, rigidity, or bradykinesia (Bernheimer et al. 1973). These conditions can be generated experimentally by dopamine depletion with reserpine, by inhibition of dopamine receptors, by mechanical or chemical ablation of nigrostriatal dopamine neurons (cut of the median forebrain bundle or injection of the toxicant 6-OH-dopamine) or the application of toxicants that leading to a relatively selective death of dopaminergic neurons in the substantia nigra (e.g. MPTP) and therefore a reduction of dopamine in the striatum (Kolata et al. 1983).

The basal ganglia include the ventral striatum, the neostriatum composed of the putamen and the caudate nucleus, the globus pallidus pars externa (GPe), the globus pallidus pars interna (GPi), the subthalamic nucleus (STN), the substantia nigra pars reticulata (SNpr) and the substantia nigra pars compacta (SNpc) (Obeso et al.2008). The main input sites into basal ganglia are the striatum and the STN where cortical (glutamatergic) innervations terminate in a topographically organized manner that largely reflects the organization in the cortex

(Fallon et al. 1978; Takada et al. 1998). Both the GPi and the SNpr represent the main output nuclei projecting into the thalamus (Parent et al.1999; Alexander et al. 1990). The connection between input and output nuclei is functionally organized into a “direct” and an “indirect” pathway (Silverdale et al. 2003). These two pathways in parallel regulate the activity of the basal ganglia output neurons of the GPi and STN and are modulated by dopamine in the striatum. The dopaminergic terminals in teh striatum originate from dopaminergic projections from the SNpc. Striatal dopamine modulates the activity of inhibitory GABAergic medium spiny neurons that make up 90% of all neurons in the striatum (Smith et al. 1994). Medium spiny neurons that preferentially express the D1 dopamine receptor are involved in the direct pathway and directly project into the two main output nuclei (GPi and SNpr). Activation of the D1 medium spiny neuronal direct pathway results in a reduction of the inhibitory basal ganglia output (GPi and SNpr) leading to a dis-inhibition of thalamic target neurons (Bolam et al.2000). These events ultimately lead to an elevated activity in the respective cortical neurons, i.e. D1 signalling in the striatum leads to an increase in motor activity.

Medium spiny neurons predominantly expressing the D2 dopamine receptor mostly project to the GPe (Gerfen et al. 1990). Activation of D2 expressing neurons leads to an inhibition of their activity. D2 neurons of the indirect pathway connect the striatum with GPi/SNpr via synaptic connections in the GPe and the STN. Activating neurons originating in the STN project into the GPi/SNpr are glutamatergic. From the STN, activating glutamatergic neuronal projections into the GPi/SNpr lead to a basal, low activation. Activation of the indirect pathway by striatal dopamine from the substantia nigra hence leads to a low basal inhibitory GABAergic output into thalamic structures, and thus allows a strong motor cortex activation of the thalamus.



(https://aopwiki.org/wiki/index.php/File:AOpicture.jpg)

Functional anatomy of basal ganglia. A) Normal conditions. Striatal (STR) dopamine mainly originates from projections originating in the substantia nigra pars compacta (SNc). The STR is mainly composed of inhibitory GABAergic medium spiny neurons (MSN). MSN involved in the direct pathway directly project to the globus pallidus pars interna (GPi) and the sunstantia nigra pars reticulata (SNpr) leading to a basal inhibition of these output nuclei. MSN involved in the indirect pathway send inhibitory projections to the globus pallidus pars externa (GPe). Their activity is dampened by dopamine binding to D2 receptor expressing MSN in the striatum. B) Lack of striatal dopamine. Under conditions of a lack of striatal dopamine, inhibitory GABAergic neurons, originating in the striatum, receive less activation, resulting in a declined inhibition of GPi and SNpr inhibitory output. In the indirect pathway, the lack of dopamine causes a lack of its inhibitory influence on inhibitory GABAergic projections into the GPe. This accelerated inhibition of the GPe results in a decline in its inhibitory output into the STN. The decline in STN inhibition allows its overactivation, resulting in an excessive activation of stimulatory glutamatergic projections into the GPi and SNpr. (according to Silverdale 2003).

Parkinson’s Disease is characterized by a decline in striatal dopamine input from the substantia nigra pars compacta (Smith t al. 1994). Under normal conditions, ganglial output via GPi/SNpr nuclei causes a moderate inhibitory influence on cortical and brainstem motor neurons. A reduction in striatal dopamine leads to an underactivation of D1 receptor-expressing medium spiny neurons of the direct pathway. This insufficient activation of the inhibitory GABAergic medium spiny neurons results in a reduction of its normal inhibitory influence on GPi and SNpr output nuclei. As a consequence, dopamine depletion leads to the overactivation of the inhibitory GABAergic GPi/SNpr output via the direct pathway (Mitchell et al.1989).

In the indirect pathway, the reduced activation of D2 receptors expressing neurons leads to an overactivation of inhibitory output nuclei projecting into the GPe. The resulting inhibitory output of the GPe is hence reduced, thus leading to a declined inhibition of the STN. Overactivation of the stimulatory glutamatergic projections originating in the STN leads to the hyperactivation of the output GPi/SNpr nuclei. As a consequence of striatal dopamine depletion, the direct pathway becomes underactivated and the indirect pathway becomes overactivated. This leads to an overactivation of the basal ganglia output nuclei. Due to their inhibitory influence on thalamocortical motor centers, the resulting reduced cortical activation leads to the prominent impairment of motor functions in parkinsonian states (Silverdale et al. 2003).

The model of direct and indirect pathways linking striatal dopamine content with the basal ganglia output nuclei has been criticized in recent years as it ignores the influence of extrastriatal dopamine (Smith et al. 2000), or the fact that some medium spiny neurons express dopamine receptors of both the D1 and of the D2 type (Surmeier et al. 1996). Principal validity of the model and the central role of striatal dopamine was e.g. demonstrated by L-DOPA-mediated supplementation of striatal dopamine content in unprimed PD patients that causes a partial reduction in the overactivation of GPi/SNpr output (Yuan et al. 2010; Heimer et al. 2006). As an alternative way for symptomatic treatment of parkinsonian conditions, deep brain stimulation of either the STN or the GPi was shown to relieve from parkinsonian motor features (Mazzone 2003, Odekerken 2013).

How it is Measured or Detected

For the analysis of striatal dopamine content and its correlation with motor control, both biochemical analysis methods on the cellular and tissue level as well as behavioral tests are required. Available test models are mice and rats on the one hand and non-human primates and humans on the other. Motor impairment features associated with parkinsonian states in man serve as reference standard. Monkey models have the advantage to largely reflect complex motor impairment patterns observed in humans which are rather difficult to assess in rodents. Rodent models in contrast are cost-efficient and allow both biochemical analysis that require major invasive methods as well as basic behavioral tests. Due to the limitations in the assessment of moderate motor impairment in rodents and the well-established correlation between striatal dopamine content and impaired motor output, analysis of striatal dopamine is often applied as surrogate readout for the assessment of motor deficits.

Detection of striatal dopamine (total or extracellular).

The standard method used in the majority of experimental work is the determination of total contents of dopamine and its two degradation metabolites HVA and DOPAC. For this purpose, the striatum is quickly removed from experimental animals, homogenized in a suitable acidic buffer, and the dopamine (metabolites) determined by HPLC with electrochemical detector or by HPLC-MS. For live in vivo detection of extracellular dopamine levels, a microdialysis probe is inserted into the striatum. Microdialysis can be performed in anesthetized animals or freely moving animals; basal dopamine levels or stimulated levels (amphetamine, KCl) can be recorded. Dopamine and its metabolites are detected in the dialysate either by HPLC or by HPLC-mass spectrometry analysis (Saraswat 1981, Cui 2009, Gonzalez 2011).

Detection of dopamine neuron terminals in the striatum.

As alternative to the detection of striatal dopamine that is to a large extent limited to live detection setups due to its instability in tissues, the number of remaining dopamine neurons in the substantia nigra pars compacta was suggested as alternative readout (Burns 1983). It allows the analysis of ex vivo samples without the limitations associated with the instability and reactivity of extravesicular dopamine. Although the number of surviving dopamine neurons in the SNpc in PD or in complex-I inhibitor challenged test animals is a valuable parameter on its own, it was discovered that the number of DA neurons in the SNpc not necessarily correlates with the amount of dopamine released in the striatum. Tyrosine hydroxylase (TH) was regularly stained as marker for DA neurons, however it was observed that TH expression was very variable following MPTP intoxication in the absence of cell death and therefore has only limited suitability for the assessment of DA neuronal numbers (Aznavour 2012). Second, many DA neurites and terminals displayed damage or degradation in the absence of death of the corresponding neuronal cell (Ling 2015). Hence, even in the presence of viable DA neurons in the SNpc, their corresponding terminals could no longer be able to release dopamine into the striatum. Staining of DA neuronal terminals in the striatum is therefore used as a more reliable indirect marker for striatal dopamine content. For the analysis of nigrostriatal terminals, the dopamine transporter (DAT) is visualized either by antibody-mediated staining in tissue slices or by the application of radioactively labeled DAT ligands that allow their application both in vivo and in ex vivo samples (Morris 1996).

Behavioral tests: Rodent models.

Rotation (https://aopwiki.org/wiki/index.php?title=Rotation&action=edit&redlink=1): the rotation model of Ungerstedt et al (Ungerstedt 1970) is based on the unilateral lesion of the nigrostriatal dopamine neuron system either in rodents or in non-human primates. The lesion can be produced either surgivally, or by stereotaxic infusion of e.g. 6-OHDA into the nigrostriatal system of one hemisphere, or by infusion of MPTP through one carotid (single sided). After the lesion, animals are left to recover, then the dopamine system is stimulated by injection of amphetamine. The asymmetry of remaining dopamine neurons (only on one side) triggers spontaneous asymmetric motor behaviour, i.e. rotations of the animals. Each full turn of an animal is recorded, the respective numbers of left- and right turns are plotted versus time, respectively. In the standard rotation

model, monkeys become hypokinetic in the limbs on the contralateral side of the brain hemisphere treated. Rats preferentially rotate towards the side of the lesion upon treatment with drugs that trigger activation of the remaining dopamine neurons.

Rotarod (https://aopwiki.org/wiki/index.php?title=Rotarod&action=edit&redlink=1): assessment of motor coordination. The animals are placed on a rotating rod that is subjected to linear acceleration. The latency to fall from the rod is detected (Jones 1968). Hang test: Detection of neuromuscular strength. Mice are placed on a horizontal grid. When the animals grabbed the grid with their fore- and hindpaws, the grid is inverted with the animal hanging upside down. In a typical setup, mice are required to remain on the grid for at least 30 s (Tillerson 2002).

Forepaw Stride length during walking (https://aopwiki.org/wiki/index.php?title=Forepaw_Stride_length_during_walking&action=edit&redlink=1). Ink is applied to the forepaws and the mice walk across a blank sheet of paper. Training of the animals to walk across the white paper in a straight line without stopping is performed before the respective treatment. The distance between single steps on each side are measured (Klapdor 1997).

Grid test (https://aopwiki.org/wiki/index.php?title=Grid_test&action=edit&redlink=1): Mice hang upside down for 30 s on the grid that is also used for the Hang test and are recorded on video for closer analysis. With this method, the average forepaw distance is measured by assessing the distance covered, divided by the number of successful forepaw steps. In the course of the analysis, the number of unsuccessful forepaw steps are detected and displayed as percentage of the total number of steps performed (Crawley 1999).

Akinesia (https://aopwiki.org/wiki/index.php?title=Akinesia&action=edit&redlink=1): the animal is placed on a flat surface and the latency until it has moved all of its four limbs is assessed. Open field test: Infrared beams detect the animals activity for the determination of parameters such as the time spent locomoting, the distance travelled, or the number of rearings.

Pole test (https://aopwiki.org/wiki/index.php?title=Pole_test&action=edit&redlink=1): the animal is placed on a gauze-taped pole with the head upwards below the top of the pole. Two parameters are detected: 1) time until animals turn by 180°; 2) time until the animals reach the floor.

Non-invasive imaging of DA neuron terminals

Positron emission tomography (PET) (https://aopwiki.org/wiki/index.php?title=Positron_emission_tomography_(PET)&action=edit&redlink=1): Based on its appropriate half life time of ca. 2 h for clinical investigations, fluorine-18 labeled L-[18F]-fluorodopa is routinely used in trace amounts for intravenous administration. Striatal uptake of L-[18F]-fluorodopa is followed by applying positron emission tomography (PET) (Leenders 1986).

Single photon emission computed tomography (SPECT) (https://aopwiki.org/wiki/index.php?title=Single_photon_emission_computed_tomography_(SPECT)&action=edit&redlink=1): monitoring of dopamine transporter (DAT). Iodine-123-β-CIT is used as a sensitive ligand for dopamine and serotonin transporters and was applied in monkeys and humans (Winogrodzka 2003).

Human neurological tests

A recent systematic review and evaluation of currently used rating scales for the assessment of motor impairment and disability in PD patients identified the 1) Columbia University rating scale, 2) the Northwestern University Disability Scale, and 3) the Unified Parkinsons Disease rating scale as the most evaluated and reliable scales available (Ramaker 2002). All scales evaluate several parameters, some of which are not motor related. Thus, only subscales are useful for readout of motor symptoms (e.g. 13 of the 42 UPDRS parameters). Of these, not all are equally dependent on nigrostriatal dopamine. Examination needs to be done by a trained neurologist.

Regulatory Examples Using This Adverse Outcome

Neurotoxic effects shall be carefully addressed and reported in routine required regulatory toxicological studies (acute toxicity studies,short-term toxicity studies, long term toxicity and carcinogenicity studies and reproductive toxicity studies). Regarding neurotoxicity in rodents, inclusion of neurotoxicity investigations in routine toxicology studies shall also be considered. For pesticide active substances the circumstances in which neurotoxicity studies should be performed are listed in Regulation (EU) No 283/2013:

Specific neurotoxicity studies in rodents shall be performed in case of one those following conditions:

- there is indication of neurotoxicity in routine toxicity studies carried out with the active substance;
- the active substance is a structurally related to known neurotoxic compound;
- the active substance has a neurotoxic mode of pesticidal action.

As a result, specific neurotoxicity studies are not routinely required for all pesticide active substances. Specific neurotoxicity testing becomes obligatory only if neurotoxicity has been observed during histopathological evaluation or in case of structural analogy with a known neurotoxic compound. Motor activity should be measured once in short-term repeated dose toxicity studies (OECD 407, 408 and 422) and several times in specific neurotoxicity studies (OECD 424, OECD 426 and cohort 2 of OECD 443). However, this is not a

requirement in chronic toxicity studies unless neurotoxic effects have been reported in the shorter studies. The same test (measures horizontal and/or vertical movements in a test chamber) is implemented in both routine studies and neurotoxicity studies. Coordination and balance are evaluated by rotation or rotarod or pole tests, and gait abnormalities by forepaw stride length test. Those tests are not required by any repeated dose toxicity OECD guidelines and they can be optionally incorporated in the design of neurotoxicity studies OECD 424 and OECD 426.

Although motor deficits is the AO in this AOP, degeneration of DA neurons, is also considered an adverse effect in the regulatory framework, even in the absence of clear clinical symptoms or motor deficits. Morphological assessment of brain structures is a standard requirement in the regulatory toxicological studies supporting the risk assessment of chemical substances and it is a regulatory expectation that the anatomical structures belonging to the nigrostriatal pathway would be included and evaluated as part of the standard evaluation of the brain. Treatment related neuronal degeneration, when occurring as a consequence of the treatment, is generally dose-dependent in incidence and severity. However, if not accompanied by clinical signs or behavioral changes indicative of central nervous system pathology, minimal loss of DA neurons would likely remain undetected in the standard histological evaluation, due to the presence of non DA neurons or as a consequence of the subjectivity of non-quantifiable analysis, unless specific markers are used. As multiple forms of perturbation can affect the neurons, some changes are potentially still reversible (e.g. loss of TH or DA) and irreversibility should be confirmed as part of the assessment. It is then important to apply a sensitive and appropriate method (Switzer 2000) and evaluation of the phenotypic markers in the striatum and in the SNpc should be always performed as a minimum standard (Minnema et al 2014) when investigating perturbation of the nigrostriatal pathway. It should additionally consider that rat is likely to be a poor model to capture this kind of hazard, as demonstrated by the poor sensitivity of rat to MPTP or related compounds and this should be taken into account for the design and interpretation of the studies.

Dissimilarities of chemical induced animal models to human disease are also important and should be carefully weighted when considering the duration and schedule of the study/treatment. Differently from the human disease, with the MPTP animal model, the damage occurs rapidly, is hardly progressive, is little age-dependent and formation of Lewy bodies is sometime not occurring (Efremova et al. 2015). Therefore, for different animals models, the standard 90 days toxicity study could not match with the chronic and progressive characteristics of the human disease and compensatory changes influencing DA metabolism and turnover and protein catabolism can occur during the treatment period with an impact on the time of onset of the lesion (Ossowska et al. 2005).

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Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
N/A, Mitochondrial dysfunction 1	directly leads to	impaired, Proteostasis	Moderate	Weak
impaired, Proteostasis	directly leads to	Degeneration of dopaminergic neurons of the nigrostriatal pathway	Strong	Moderate
N/A, Neuroinflammation	directly leads to	Degeneration of dopaminergic neurons of the nigrostriatal pathway	Moderate	Moderate
Degeneration of dopaminergic neurons of the nigrostriatal pathway	directly leads to	N/A, Neuroinflammation	Moderate	Moderate
N/A, Mitochondrial dysfunction 1	indirectly leads to	Degeneration of dopaminergic neurons of the nigrostriatal pathway	Moderate	Weak
Degeneration of dopaminergic neurons of the nigrostriatal pathway	directly leads to	Parkinsonian motor deficits	Strong	Strong
Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I)	directly leads to	Inhibition, NADH-ubiquinone oxidoreductase (complex I)	Strong	Weak
Inhibition, NADH-ubiquinone oxidoreductase (complex I)	directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Moderate

N/A, Mitochondrial dysfunction 1 leads to impaired, Proteostasis (https://aopwiki.org/relationships/904)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

The ubiquitin proteasome system is highly conserved in eukaryotes, from yeast to human. Ubiquitin is a small (8.5 kDa) regulatory protein that has been found in almost all tissues of eukaryotic organisms. For instance, drosophila has been used as PD model to study the role of ubiquitin in α-synuclein induced-toxicity (Lee et al., 2009). Human and yeast ubiquitin share 96% sequence identity. Neither ubiquitin nor the ubiquitination machinery are known to exist in prokaryotes. Autophagy is ubiquitous in eukaryotic cells and is the major mechanism involved in the clearance of oxidatively or otherwise damaged/worn-out macromolecules and organelles (Esteves et al., 2011). Due to the high degree of conservation, most of the knowledge on autophagy proteins in vertebrates is derived from studies in yeast (Klionsky et al., 2007). Autophagy is seen in all eukaryotic systems, including fungi, plants, slime mold, nematodes, fruit flies and insects, rodents (i.e., laboratory mice and rats), and humans. It is a fundamental and phylogenetically conserved self-degradation process that is characterized by the formation of double-layered vesicles (autophagosomes) around intracellular cargo for delivery to lysosomes and proteolytic degradation.

How Does This Key Event Relationship Work

In any cell type, including neurons, the protein homeostasis (proteostasis) plays a key role in cellular functions. There are two major systems involved in the removal of damaged cellular structures (e.g. defective mitochondria) and misfolded or damaged proteins, the ubiquitin-proteasome system (UPS) and the autophagy–lysosome pathway (ALP). These processes are highly energy demanding and highly susceptible to oxidative stress. Upon mitochondrial dysfunction UPS and ALP functions are compromised resulting in increased protein aggregation and impaired intracellular protein/organelles transport (e.g. Zaltieri et al., 2015; Song and Cortopassi, 2015; Fujita et al., 2014; Esteves et al., 2011; Sherer et al., 2002).

Weight of Evidence

The weight of evidence supporting the relationship between mitochondrial dysfunction and impaired proteostasis, including the impaired function of UPS and ALP that results in decreased protein degradation and increase protein aggregation is strong.

Biological Plausibility
The biological relationship between Mitochondrial dysfunction and Impaired proteostasis (unbalanced protein homeostasis) that involves dysregulation of proteins degradation (misfolded or damaged) as well as removal of cell organelles is partly understood. Under physiological conditions, mechanisms by which proteostasis is ensured include regulated protein translation, chaperone assisted protein folding and functional protein degradation pathways. Under oxidative stress, the proteostasis function becomes burdened with proteins modified by ROS (Powers et al., 2009; Zaltieri et al., 2015). These changed proteins can lead to further misfolding and aggregation of proteins (especially in non-dividing cells, like neurons). Particularly in DA cells, oxidative stress from dopamine metabolism and dopamine auto-oxidation may selectively increase their vulnerability to CI inhibitors (such as rotenone) and cause additional deregulation of protein degradation (Lotharius and Brundin, 2002; Esteves et al., 2011). As most oxidized proteins get degraded by UPS and ALP (McNaught and Jenner, 2001), mitochondrial dysfunction and subsequent deregulation of proteostasis play a pivotal role in the pathogenesis of PD (Dagda et al., 2013; Pan et al., 2008; Fornai et al., 2005; Sherer et al., 2002). It is also well documented that increased oxidative stress changes the protein degradation machinery and leads to a reduction of proteasome activity (Lin and Beal, 2006; Schapira, 2006).

Empirical Support for Linkage
Based on the existing in vitro and in vivo data it is suggested that mitochondrial dysfunction impairs protein homeostasis through oxidative and nitrosative stress resulting in protein aggregation, disruption of microtubule assembly and damaged intracellular transport of proteins and cell organelles.

Mitochondrial dysfunction by rotenone or MPP+ reduces UPS activity:

- Mitochondrial dysfunction induced by systemic and chronic CI inhibition by rotenone, results in a selective inhibition of proteasomal function in the midbrain (not in cortical or striatal homogenates) of rats that had lost the TH-positive terminals in the striatum. Initially, proteasomal activity showed an acute increase prior

to a decrease by 16-31 %, during chronic rotenone exposure (3.0 mg/kg/day, through osmotic pump during 5 weeks). In the same animals a significant and selective increase in ubiquitinated proteins was observed (~ 25%) in the ventral midbrain of lesioned rats, indicating an increase in the proteins levels that have been marked for degradation by UPS. These results were confirmed immunocyto-chemically, pointing out that ubiquitin levels were elevated selectively in DA neurons present in SNpc (Betarbet et al., 2006).

- Nigral neurons in chronically rotenone-treated rats (up to 5 weeks, infusion of rotenone at 2.5 mg/kg/day) accumulate fibrillar cytoplasmic inclusions that contain ubiquitin and α-synuclein (the main protein of Lewy bodies observed in PD) (qualitative data, obtained by immuno-electron microscopy) (Betarbet et al., 2000).
- Inhibition of proteasomal function was also observed in in vitro systems using SK-N-MC human neuroblastoma. Exposure to 5 nM rotenone, for up to 4 weeks caused 60% increase in the levels of ubiquinated proteins, suggesting that chronic exposure to rotenone increased the level of misfolded or oxidized proteins targeted for degradation by UPS (Betarbet et al., 2006).
- To determine whether rotenone-induced proteasomal inhibition was due to CI inhibition or direct effects of rotenone on the UPS, proteasomal activity was determined in SKN-MC cells expressing the rotenone-insensitive single-subunit NADH dehydrogenase of Saccharomyces cerevisiae (NDI1), which acts as a "replacement" for the entire CI in mammalian cells (Bai et al., 2001; Seo et al., 2000, 2002). The obtained results confirmed that rotenone-induced proteasomal dysfunction is due to CI inhibition and not to direct effects of rotenone on proteasomal function (Betarbet et al., 2006). In the same study the decreased proteasomal activity and an accumulation of ubiquitinated proteins was completely prevented by continuous treatment with α-tocopherol (62.5 µM added 1 week prior to and continuously thereafter along with 5 nM rotenone) (qualitative data), confirming that oxidative damage played a major role in rotenone-induced proteasomal dysfunction rather than bioenergetic defects. Indeed, chronic, low levels of rotenone exposure did not changed significantly ATP levels (111.5 ± 1.5% of control), but produced ROS (not shown in this study). Similar results were published by Shamoto-Nagai's group (Shamoto-Nagai et al. 2003).
- Rotenone significantly lowered UPS activity in a concentration dependent manner in HEK (human embryonic kidney cells) and SK-N-MC human neuroblatoma cells even after 24 h exposure to doses as low as 10 nM. It caused a reduction in the 20S proteasome activity (by 5-25%) and of the 20S proteasome subunit (by 20-60%) (as shown by increase of GFP-U fluorescence) (Chou et al., 2010). Similar results were obtained using other pesticides that inhibit CI, including pyridaben and fenazaquin (Wang et al., 2006). This effect was mediated by oxidative stress as anti-oxidants, such as butylated-hydroxy toluene (BHT), and catalase attenuated rotenone-induced UPS inhibition. Additionally, nitric oxide (NO) and peroxinitrite contributed to this effect as well, since neuronal nitric oxide synthase (nNOS) inhibitor (LNMMA) attenuated rotenone-induced proteasome inhibition by 20% (Chou et al., 2010) indicating that both oxidative and nitrative stress can directly inhibit the proteasome activity through increased degradation of proteasome subunits. The same mechanisms of proteasome inhibition were suggested by many other studies (e.g. Szweda et al, 2002; Osna et al., 2004; Shamoto-Nagai et al., 2003).
- CI inhibition-induced proteasomal dysfunction has been reported in ventral mesencephalic cultures following acute rotenone or MPP+ exposure (Hoglinger et al., 2003). In DA neurones derived from rat (embryonic day 15.5) ventral mesencephalon, it has been showen that proteasome inhibition (by 100 nm epoxomicin) exacerbated the neurotoxicity of CI inhibitors (by mean of rotenone 30 nM, or MPP+ 3 µM, for 24 hr). All three proteasomal peptidase activities (i.e., chymotrypsin (CT)-like, trypsin (T)-like, and peptidylglutamyl-peptide hydrolase (PGPH) activity) significantly decreased in cultures upon 6 hr treatment with 30 nM rotenone (by 50+-60%) or 30 µM MPP+ (by 25-30%) (Hoglinger et al., 2003).
- CI inhibition-induced proteasomal dysfunction has been reported in human SH-SY5Y neuroblastoma cells following acute rotenone exposure (Shamoto-Nagai et al., 2003). After 96 hr of incubation with 25 or 50 nM rotenone, the activity was reduced respectively to 28.7% and 21.9% of control, and adding ATP did not increase the activity. After 120 hr, the activity was virtually undetectable (with or without added ATP). On the contrary, the levels of the proteins composing proteasome did not change with rotenone treatment (Shamoto-Nagai et al., 2003).
- The ability of rotenone to cause proteasome inhibition via disruption of microtubules (MT) assembly has been also documented. In human embryonic kidney (HEK) and neuroblastoma SK-N-MC cells rotenone (10-100-100 nM, 24 hr) was found to inhibit 26S UPS activity (by 25%, at 10 nM) (Chou et al., 2010). Rotenone was found to interfere with MT assembly at concentrations as low as 10 nM, providing evidence that there could be additional mechanisms implicated in the rotenone induced UPS inhibition, possibly mediated by nitric oxide (NO). In the same study, nocodazole, a MT disrupter (positive control), strongly inhibited the UPS activity (e.g., 10 µM nocodazole caused ~80% decrease of 26S UPS activity) (Chou et al., 2010).

• Oxidative stress triggered by the MPP+ inhibited CI (1 mM, for 2-6-24 hr) led to a decrease in proteolytic activity, as shown in NT2 human teratocarcinoma cells containing mitochondrial DNA (ρ+) and NT2 cells depleted of mtDNA (ρ0) (Domingues et al., 2008). In particular, MPP+ (1 mM, 2 hr) elevated ubiquitinylated

protein content (by ~3 fold compared to untreated Ctr), and after 24 hr induced a significant decrease of chymotrypsin-like activity (by ~30%) and peptidyl-glutamyl peptide hydrolytic-like activity (by ~75%) compared to untreated cells (Domingues et al., 2008).

- Mice following continuous MPTP infusion (1-5-30 mg/kg daily) exhibited inhibition of the UPS (respectively by 40-50-60%) and increased inclusions of ubiquitin and α-synuclein in the neurons in the substantia nigra (Fornai et al., 2005).
- A mouse model of mitochondrial CI deficiency (Ndufs4-/- mice) showed an impaired 20S proteasomal activity (by ~50%), leading to increased ubiquitin protein levels (by ~40%) in the substantia nigra (not in cortex and hippocampus), increased of ubiquitin+/TH+ neurons (by ~2 fold, compared to WT mice), and increased ubiquitinated neurofilaments in the midbrain (values of 1.2 - 2.8 vs 1.0 in WT) (Song and Cortopassi, 2015).

Human studies.

- PD patients appear to have an impaired UPS. The presence of aggregated, poly-ubiquitinated proteins in Lewy Bodies indicates that proteolytic dysfunction and proteo-toxicity are critical steps in the pathogenic cascade of PD (Betarbet et al., 2005). In this regard, impairment of proteasomal activity and reduced expression of proteasomal subunits have been reported selectively in subtantia nigra of sporadic PD post-mortem brains (McNaught et al., 2003; McNaught and Jenner, 2001). In particular, in PD, there was a 40.2% reduction in the amount of α-subunits in the SNc. On the opposite α-subunits levels were increased by 9.2% in the cerebral cortex and by 29.1% in the striatum in PD compared to Ctr (McNaught et al., 2003). Chymotrypsin-like, trypsin-like, and peptidyl glutamyl-peptide hydrolytic (PGPH) 20/26S proteasomal activities were significantly decreased in the substantia nigra (by 43.9%, 45.9%, and 44.6% respectively) (not in the cortex or striatum) in PD patients. At the same time, in PD there was a marked increase in the levels of PA700 subunits (the 19S regulatory complex of the 26S proteasome) in the frontal cortex and/or the striatum compared to controls, while in the SNpc PA700 subunits resulted decreased up 33%, whereas levels of nigral PA28 were almost undetectable in both normal and PD subjects (McNaught et al., 2003).
- Steady-state levels of soluble AF-6 (modulates parkin ubiquitin-ligase activity) have been found significantly lower in the caudate/putamen (~66% lower) as well as in the SN of PD patients (~66% lower). AF-6 was also detected in ~25% of mature Lewy bodies and in occasional Lewy neurites in the substantia nigra of the four PD brains analysed, and may contribute to the disruption of mitochondrial homeostasis (Haskin et al. 2013).
- HDAC6 has recently been identified by immunocytochemistry as a constituent in Lewy bodies of PD and dementia with LBs (DLB), as well as in glial cytoplasmic inclusions in multiple system atrophy (MSA) (Kawaguchi et al. 2003; Miki et al. 2011; Chiba et al. 2012). HDAC6 is considered a sensor of proteasomal inhibition and a cellular stress surveillance factor. Upon proteasomal inhibition, HDAC6 is relocated and recruited to polyubiquitin-positive aggresomes. HDAC6 inhibition elicits tubulin acetylation and restores microtubule (MT)-dependent transport mechanisms in neurons (Richter-Landsberg and Leyk, 2013).
- Basal activity of 20S proteasome was significantly reduced (by ~33%) in PD as compared to control fibroblasts. Higher accumulation of ubiquitinated proteins (by ~2 fold), representative of impaired 26S proteasome function, were found in PD as compared to Ctr cells at baseline. In the presence of rotenone (20 and 500 µM, 6 hr) PD-derived fibroblasts showed a higher induction of 20S proteasome activity (~15% higher) as compared to Ctr fibroblasts, with no significant changes in autophagy (except from increased LC3-II accumulation in both groups after exposure to 500 µM rotenone) (Ambrosi et al., 2014).

Mitochondrial dysfunction by rotenone or MPP+ deregulates ALP activity

- Exposure to rotenone (10 µM, 24 hr) induced neurotoxicity in human neuronal SH-SY5Y cells (number of dead cells was 8 folds higher than Ctr group) and pre-treatment with rapamycin (3 µM, 48 hrs) (strong inducers of autophagy) robustly protected against rotenone-mediated toxicity (number of dead cells was 3 folds higher than Ctr group) and this was due to the induction of autophagy. Indeed, suppression of autophagy (by silencing of Atg5) blocked the neuroprotection of rapamycin (Pan et al., 2009).
- Similar results were produced using kaempferol (6 µM, 1 hr prior addition of rotenone) and rotenone (50 nM, max up to 24 hr) on SH-SY5Y cells. Kaempferol was found to counteract rotenone-induced effects (see KER2) and these protective effects were related to induction of autophagy (6 hr kaempferol induced LC3-II formation, as shown by Western blot) (Filomeni et al., 2012).
- Treatment of SH-SY5Y cells with high doses of rotenone (500 nM, 48 hr) induced Atg5–Atg12 dependent autophagy, which leads to lysosomal dysfunction, increased p62 levels, and an aberrant accumulation of α-synuclein (Pan et al., 2009; Dadakhujaev et al., 2010). In particular, in α-synuclein expressing SH-SY5Y cells Atg5–Atg12 were increased by addition of rotenone and rapamycin (100 nM, 48 hr). Co-treatment with rotenone and autophagy inhibitors (e.g., 3-MA, bafilomycin or wortmannin) similarly diminished the level of Atg5–Atg12 in α-synuclein expressing cells (western blot analyses) (Dadakhujaev et al., 2010).
- A few studies have suggested that rotenone can act as an inducer of autophagic flux. For instance,

treating human embryonic kidney cells (HEK 293) and U87 glioma cells with rotenone (50 µM, for 0-72 hr) caused cell death (in HEK 293 cells, rotenone induced 30% cell death, after 72 hr; in U87 cells, 40%) by upregulating autophagy and mitophagy (as shown by increase of cells with AVOs (indicative of autophagosomes and autolysosomes, analysed by flow cytometry): by ~14% in HEK 293 cells, and by ~20% in U87 cells, as compared to untreated cells, 0%), a process that is supposed to be triggered by mitochondrial superoxide (Chen et al., 2007).

- Increased autophagic flux has been observed in SH-SY5Y cells and primary cortical neurons treated respectively with 1 µM and 250 nM of rotenone. Rotenone elicited increases in autophagy (~ 2 folds vs Ctr) and mitophagy (i.e., as shown by the percentage of GFP-LC3 puncta colocalizing with mitochondria (~ 4 folds vs Ctr), indicating a preferential increase in “mitophagosomes” relative to total autophagosomes. Additionally, rotenone induced a decrease in p62 (SQSMT1), levels (~40% decrease with 250 nM), consistent with increased autophagic flux. This effect was reversed by co-treating cells with bafilomycin A2, a specific inhibitor of vacuolar-type H(+)-ATPase, or by RNAi (knockdown of ATG7 and ATG8/LC3). The mechanism by which LC3 recognizes damaged mitochondria in rotenone-treated neurons involves, among others, the externalization of cardiolipin and recruitment of LC3 at the mitochondria initiating rotenone induced-mitophagy and lysosomal-mediated degradation of mitochondria (Chu et al., 2013).
- In the study by Wu et al., (2015) chronically rotenone-treated rats (male Lewis rats received rotenone 1mg/kg subcutaneously twice a day for 8 weeks) had a robust loss of TH+ neurons in striatum (~50%) and in SNpc (~30%). However, in the remaining DA neurons of SNpc, cytoplasmic inclusions containing α-synuclein were observed (~7% of α-synuclein+/TH+ cells vs ~2% in Ctr), probably due to rotenone-induced decreased degradation of the autophagosomes (upregulation of LC3-II by ~30%, Beclin 1 by ~10%, and p62 by ~150%, after 24 hr rotenone) indicating decreased ALP function. Compared with the control group, the nigral DA neurons of the rotenone-treated group exhibited an increased diffuse distribution of LAMP2 (~15% vs ~25% Ctr) and cathepsin D (~22% vs ~60% Ctr) instead of punctuate pattern, indicating impaired lysosome integrity and a redistribution of cathepsin D from lysosomes to the cytosol. In parallel in vitro studies by the same group showed that PC12 cells exposed to rotenone (500 nM for 24 hr) underwent increased protein levels (but not mRNA levels) of α-synuclein (~4.5 folds vs Ctr), indicating an impairment of protein degradation. In TEM pictures, the majority of neurons displayed mitochondrial swelling, crista fragmentation, and accumulation of double membrane structures containing damaged mitochondria, which were stalled autophagosomes (Wu et al., 2015).

Similar results, showing impaired autophagic flux resulting in α-synuclein accumulation and the rupture of lysosomes in neuronal cell lines exposed to rotenone have been described in many other studies (e.g. Mader et al., 2012; Sarkar et al., 2014).

- Rotenone produced bidirectional effects on macroautophagy (decrease or increase). This may be attributed to differences in the dosage, the duration, and cell type which can produce variable levels of ROS and mitochondrial damage (Pan et al., 2009; Dadakhujaev et al., 2010; Chen et al., 2007; Filomeni et al., 2012; Mader et al., 2012).
- MPP+ (2.5 mM, 24 - 48 hr) increased autophagy (~14 folds increase vs Ctr, of LC3-II) and mitochondrial loss in SH-SY5Y cells (a DA neuronal cell line widely used as a cell culture model of PD) by increased MAP kinase signalling (MEK inhibition by UO126 reversed by both autophagy and mitochondrial loss elicited by MPP+) (Zhu et al., 2007).
- Another study from the same group showed that longer MPP+ treatment (250 µM, 2 weeks) induced formation of enlarged, coarse GFP-LC3 puncta, in a time- and dose-dependent manner (~1.8% of cells presenting coarse GFP-LC3 puncta, vs ~0.2% in Ctr, at 14 days with 250 µM rotenone) (Zhu et al., 2012).
- An in vitro study on MN9D cells (a fusion of embryonic ventral mesencephalic and neuroblastoma cells, used as a model of DA neurons) showed that MPP+ (50 µM, for 24 hr) blocked autophagic flux, as evidenced by increased steady-state levels of p62 (qualitative data, Western blot), increased of authophagic vacuoles numbers (~3 folds vs Ctr) along with lysosomal depletion and dysfunction presumably due to leakage of lysosomes, impaired lysosomal biogenesis, and increased proteasomal-mediated degradation of proteins (as shown by time-dependent increase of ubiquitinated proteins, by IC) (Lim et al., 2011).
- In another study human neuroblastoma BE-M17 cells were treated with MPP+ (0.25-2.5 mM, 24 hr); Lamp1 protein levels were decreased in a dose-dependent manner in MPP+-treated cells (by ~40% at 2.5 mM), without concomitant decreases in mRNA expression levels. Also, LC3-II increased in a dose-dependent manner with MPP+ treatment (~3000% increase at 2.5 mM vs Ctr), indicating lysosome depletion and autophagosome accumulation upon MPP+ treatment. These data were confirmed in vivo: lysosomal depletion and accumulation of autophagosomes (as shown by ~600% increase of LC3-II, and ~40% decrease of Lamp1, after 1 day of MPTP injection compared to saline) occurred also in MPTP-intoxicated mice (30 mg/kg/day, for 5 consecutive days) (Dehay et al., 2010).
- Other in vivo data support a negative role of MPTP on autophagic flux. Mice were i.p. injected with 2 mg/ml MPTP (30 mg/kg) for 7 days. Suppression of autophagic flux induced by MPTP (~20% reduction vs Ctr) was detrimental to neuronal survival (as shown by ~60% decrease of TH+ neurons). Treating mice with the autophagy inducer rapamycin after seven days of MPTP treatment (daily i.p. injections of 2 mg/ml MPTP (30 mg/kg) for 7 days, followed by 0.1 ml of 20 µg/ml rapamycin by i.v. for an additional 7

days), significantly increased the number of surviving dopamine neurons (~60% TH+ neurons vs ~30% with MPTP alone, as compared to Ctr 100%) and the levels of TH protein (~75% vs ~60% with MPTP alone, as compared to Ctr 100%) and decreased the levels of α-synuclein aggregates (~210% of α-synuclein protein level, vs ~300% with MPTP alone, as compared to Ctr 100%) (Liu et al., 2013).

- Treating mice with the autophagy inducer rapamycin after seven days of MPTP treatment (daily i.p. injections of 2 mg/ml MPTP (30 mg/kg) for 7 days, followed by 0.1 ml of 20 μg/ml rapamycin by i.v. for an additional 7 days), significantly increased the number of surviving dopamine neurons (~75% of TH protein level vs ~60% with MPTP alone) and decreases the levels of α-synuclein aggregates (~210% of α-synuclein protein level, vs ~300% with MPTP alone) (Liu et al., 2013).

MPP+ induced dysregulation of macroautophagy in neurons is discussed in recently published reviews (e.g. Cherra et al., 2010; Jiang et al., 2010). The potential other mechanisms by which rotenone or MPTP induce mitochondrial dysfunction are further discussed in recent publications (e.g. Dagda et al., 2013; Esteves et al., 2011).

Impaired UPS and ALP function leads to α-synuclein aggregation:

α-synuclein is one of the most abundant neuronal proteins (Vekrellis et al., 2011). Several PD-related mutations and environmental toxicants cause autophagy dysfunction and lead to the accumulation of misfolded proteins in DA neurons, including α-synuclein. Both monomeric and aggregated forms of α-synuclein can be degraded by macroautophagy, whereas only wild-type α-synuclein (not Ala30Pro, Ala53Thr and Glu46Lys mutant forms) is degraded by the process of chaperone-mediated autophagy (CMA) (Vekrellis et al., 2011).

- Rotenone-induced α-synuclein aggregation has the ability to inhibit proteasome activity due to its propensity to assemble into filaments (as reviewed in Zaltieri et al., 2015). In particular, expression of α-synuclein was found to inhibit proteasome activity in SH-SY5Y cells. Increased levels of GFP-CL1 band were observed in cells coexpressing GFP-CL1 and α-synuclein (~9000 arbitrary units (au) vs ~500 au in DMSO-Ctr), indicating that proteasome activity is inhibited effectively by expression of α-synuclein (Nonaka and Hasegawa, 2009).
- By using stable PC12 cell lines expressing wild-type (WT) or A53T mutant human α-synuclein it has been shown that cells expressing mutant α-synuclein showed: (1) disruption of the ubiquitin-dependent proteolytic system, manifested by small cytoplasmic ubiquitinated aggregates and by an increase in polyubiquitinated proteins (qualitative data); (2) marked accumulation of autophagic-vesicular structures (qualitative data); (3) reduction of lysosomal hydrolysis and chymotrypsin-like proteasomal function (by ~ 30%, compared to WT) (Stefanis et al., 2001).
- Rotenone- (or MPP+)-induced inhibition of CI results in calcium (Ca2+) release from mitochondria. Calcium rise and oxidative stress cooperatively can promote α-synuclein aggregation (Follett et al., 2013; Goodwin et al., 2013; Nath et al., 2011).
- For instance, to investigate the influence of raised Ca2+ in response to plasma membrane depolarization on the aggregation of a-synuclein, HEK293T and SH-SY5Y neuroblastoma cells have been used and depolarized by addition of KCl to the cell culture medium. After KCl treatment (50 mM) increase of cellular Ca2+ was observed (~90% increase 20 min after KCl treatment), leading to the formation of frequent perinuclear α-synuclein focal aggregates at 26–74 hr post-treatment (qualitative IC images). By adding TMO (a selective T-type Ca2+ channel blocker) no a-synuclein aggregates were detected (Follett et al., 2013).
- Similarly, increased intracellular free Ca2+ (obtained by treating cells with either calcium ionophore or thapsigargin) induced the formation of α-synuclein aggregates in α-synuclein-GFP-transfected 1321N1 glioma cells (~65% increase compared to Ctr-untreated cells) (Nath et al., 2011).
- On the other hand, α-synuclein can control mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions. Silencing of endogenous α-synuclein (siRNA-α-syn) in HeLa cells was found to impair mitochondrial Ca2+ transients (~35% decrease compared to Ctr-scrambled siRNA) and morphology (Calì et al., 2012). Also, α-synuclein oligomerization exacerbates calcium dysregulation by increasing mitochondria permeability transition (Danzer et al., 2007). Therefore, it is possible that mitochondrial dysfunction-induced calcium rise precede the onset of α-synuclein accumulation leading to UPS inhibition (Chou et al., 2010).
- It has been demonstrated that rotenone increased the intracellular calcium levels, triggering aggregation and phosphorylation of α-synuclein in a calcium-dependent manner. The aggregation of α-synuclein in PC12 cells following rotenone exposure was observed in a dose and time-dependent manner (1, 10 and 100 nM for 48 hrs, 3 days, 1 and 3 weeks) (~4 fold increase of α-syn with 100 nM rotenone for 48 hr, vs Ctr; and also, ~2.5 fold increase of α-syn with 1 nM rotenone for 1 week, vs Ctr) as evaluated via a variety of methods, including western blotting, immunofluorescence and electron microscopy. The observed attenuation of autophagy and α-synuclein aggregation was reversed by scavenging calcium (by using the calcium chelator BAPTA at 10 μM). Aggregated α-synuclein is typically degraded by autophagy, but rotenone impaired this process (Yuan et al., 2015).

- Under physiological conditions, α-synuclein is degraded by both the proteasome and autophagy. Mutant α-synuclein inhibits ALP functioning by tightly binding to the receptor on the lysosomal membrane for autophagy pathway control (e.g. Pan et al., 2009; Betarbet et al., 2000).
- The strongest evidence supporting that mitochondrial dysfunction precedes the onset of α-synuclein pathology derives from studies on rotenone and MPTP in which repetitive exposure of rodents and monkeys to these chemicals via oral, intraperitoneal, intragastric, or nasal administration resulted in the pathological accumulation of α-synuclein in central as well as peripheral neurons (Cannon et al., 2009; Drolet et al., 2009; Mandel et al., 2004; Pan-Montojo et al., 2012 and 2010; Tristão et al., 2014). For example, male Lewis rats were injected with rotenone (2.0 mg/kg, i.p.) and sacrificed at 0, 4, 8, 16, or 32 h after injection and showed α-synuclein and poly-ubiquitin accumulation and aggregation (as shown by IHC data) (Cannon et al., 2009).

Drolet and colleagues injected rats with rotenone (2.0 mg/kg, 1.0 ml/kg, i.p. 5 injections/week for 6 weeks) and found formic acid-resistant α-synuclein aggregates in the small intestine myenteric plexus, particularly 6-months after the last rotenone injection (3.5 median, vs 2.0 in Ctr) (Drolet et al., 2009). Mandel et al. injected male C57-BL mice with MPTP (24 mg/kg/day, ip for 5 days) and found α-synuclein aggregates (IHC data), which were decreased by using the radical scavengers apomorphine (injected s.c. at 10 mg/kg/day) or epigallocatechin-3-gallate (EGCG, given alone orally, 2 mg/kg/d) for 10 days) or a combination of both (Mandel et al., 2004).

Inhibition of the mitochondria respiratory chain induces oxidative stress that in turn leads to lipid peroxidation of cellular and vesicular membranes at synaptic sites, resulting in dysfunction of neurotransmitter release. These effects facilitate α-synuclein conformational changes, such as accumulation, and aggregation. It has been demonstrated that synaptic dysfunction (caused by mitochondrial dysfunction) triggered the accumulation of α-synuclein (Nakata et al., 2012). Also, alterations of mitochondrial fission or dynamics can reduce synaptic mitochondrial load and impair neuronal function by hindering the proper energy demand to ensure synaptic function. Mitochondrial behaviours, especially those regulated by neuronal activity and synapse location, determine their distribution in the axon (Obashi and Okabe, 2013). These observations support the idea that mitochondrial dysfunction can affect synaptic environment and consequently result in α-synuclein accumulation at synapses (Zaltieri et al., 2015).

- It was found that continuous administration of MPTP produced formation of nigral inclusions immunoreactive for ubiquitin and α-synuclein (Fornai et al., 2005). Mice were implanted with osmotic pump to deliver MPTP-HCl. Delayed and prolonged inhibition of striatal proteasome activity (i.e., 40-50-60% inhibition of UPS) occurred after continuous MPTP administration (respectively, 1-5-30 mg/kg MPTP daily) for the indicated time periods (Fig. 1) (Fornai et al, 2005). Continuous MPTP infusions caused also a long-lasting activation of glucose uptake. Additionally, in mice lacking α-synuclein, the MPTP-induced inhibition of the UPS system and the production of inclusion bodies were reduced (e.g., Ctr mice showed ~40% inhibition of postglutamyl peptidase (PGPH) activity, vs ~13% inhibition observed in α-synuclein KO mice) (Fig. 2), suggesting that α-synuclein could play an important role in UPS inhibition induced by MPP+ (Fornai et al., 2005). These data suggest that continuous, low-level exposure of mice to MPTP causes a Parkinson-like syndrome in a α-synuclein-dependent manner (Fornai et al., 2005).

These results are supported by other studies showing that α-synuclein–/– mice are resistant to MPTP toxicity (Dauer et al., 2002; Drolet et al., 2004). MPTP exposure (0.5, 5, 50 μM, 48 hr) increases in a dose-dependent manner the α-synuclein protein level in mesencephalic neurons in culture (e.g., ~70% increase at 5 μM vs Ctr) (Duka et al., 2006). Increased expression of α-synuclein predisposes DA neuronal cells to proteasomal dysfunction (~50% decrease compared to Ctr-vector cells) (Sun et al., 2005).

- Accumulation/overexpression of α-synuclein, both wild type and mutant, potentiates inhibition of proteasomal activity. Cells expressing mutant α-synuclein showed a reduction of lysosomal hydrolysis and chymotrypsin-like UPS function (by ~30%, compared to WT) (Stefanis et al., 2001).
- Proteasomal inhibition (by mean of lactacystin, a proteasome inhibitor, used at different concentrations for 24 hr) contributes to the accumulation of α-synuclein as it has been described by immunostaining in PC12 cells (Rideout et al., 2001) and in primary mesencephalic neurons (McNaught et al., 2002).
- α-Synuclein levels were selectively increased in the ventral midbrain (VMB) region of rotenone-infused rats with or without lesion (~ 110% increase vs Ctr) (Fig. 3) (Betarbet et al., 2006). Rotenone was administered up to 5 weeks, at 2.5 mg/kg/day. Additionally, 4 weeks of in vitro rotenone exposure (5 nM, on SK-N-MC human neuroblastoma cells) increased α-Synuclein levels by 24%, while lactacystin (9 μM, overnight) did not induce any detectable changes in α-synuclein levels. α-Tocopherol attenuated the rotenone-induced increase in α-synuclein (comparable to Ctr) (Fig. 4). Furthermore, levels of ubiquitinated proteins detected in solubilized protein fractions from SK-N-MC cells resulted increased (by 60%) with rotenone treatment (5 nM), and even more (by 484%) with rotenone combined with lactacystin (Fig. 5) (Betarbet et al., 2006).
- CI inhibition-induced proteasomal dysfunction has been reported in human SH-SY5Y neuroblastoma cells following acute rotenone exposure (Shamoto-Nagai et al., 2003). The proteasome activity decreased in the cells treated with rotenone (25 or 50 nM) in a time- and dose-dependent way. ATP addition restored the reduction of proteasome activity in the cells treated with 25 nM rotenone for 72 hr. However, after 96 hr of incubation with 25 or 50 nM rotenone, the activity was reduced respectively to 28.7% and 21.9% of control, and adding ATP did not increase the activity. After 120 hr, the activity was virtually undetectable

(with or without added ATP) (Fig. 6). On the contrary, the levels of the proteins composing proteasome did not change with rotenone treatment (Shamoto-Nagai et al., 2003).

Cytoskeletal damage further enhances disturbed proteostasis:

- α-synuclein can trigger hyperphosphorylation of Tau. Treatment of primary mesencephalic neurons acutely (48 h) or subchronic treatment of wild-type (WT) mice with MPP+/MPTP results in selective dose-dependent hyperphosphorylation of Tau at Ser396/404 (p-Tau). The presence of α-synuclein was absolutely mandatory to observe MPP+/MPTP-induced increases in p-Tau levels, since no alterations in p-Tau were seen in transfected cells not expressing α-synuclein or in α-synuclein-/- mice. MPP+/MPTP also induced a significant accumulation of α-synuclein in both mesencephalic neurons and in WT mice striatum. Sub-chronic MPTP exposure increased phosphorylated-Tau in striatum of WT (but not α-Syn-/- mice) causing microtubule (MT) cytoskeleton instability that affects cellular microtubule transport (including axonal transport) (Qureshi et al., 2009; Duka et al., 2006). For instance, MPTP was found to elicit an increase of phosphorylated Tau at Ser262 by 2.8-, 4.5-, 4.6-, and 4.0-fold higher in 1, 5, 25, and 50 μM MPTP-treated cells than the basal level observed in Ctr/vehicle-treated cells, respectively. Additionally, MPTP caused a dose-dependent increase in the intracellular α-synuclein level in M17 human neuroblastoma cells (~3.5 fold increase in cells treated with 25 μM MPTP vs Ctr) (Qureshi and Paudel, 2009). These results were confirmed by other studies (e.g. Dauer et al., 2002; Drolet et al., 2004 etc.).
- α-synuclein accumulation followed by MT depolymerisation induces disruption in axonal transport, which leads to an accumulation of damaged organelles, aggregated/misfolded proteins and impaired vesicular release. Dopamine is leaking from the vesicles to the cytosol promoting an increase in oxidative stress, potentiated by dopamine oxidation (Feng, 2006; Kim et al., 2007). When microtubule network is disrupted, the amount of free tubulin increases, triggering α-synuclein fibrillization (Payton et al., 2001).
- Axonal transport might be impaired by misfolded α-synuclein through perturbation of microtubule assembly (Esposito et al., 2007; Lee et al., 2002; Chen et al., 2007), especially together with MAPT protein (Qureshi and Paudel, 2011; Giasson et al., 2003). It induces not only microtubule disruption but also impairs microtubule-dependent trafficking (Lee at al., 2006). MT-dependent transport is important for maintaining the Golgi structure, and thus, depolymerization of the MT leads to a specific pattern of Golgi fragmentation (Cole et al., 1996). When the MT network was disrupted by nocodazole treatment (5 μg/mL) or α-synuclein was overexpressed, this normally compact organelle was fragmented and dispersed (IC images) as shown in COS-7 cells (Lee at al., 2006). Similarly, overexpression of α-synuclein in differentiated SH-SY5Y cells caused Golgi fragmentation (e.g., ~190% increased fragmented Golgi at 12 m.o.i. (multiplicity of infection) of α-synuclein vs Ctr) (Lee at al., 2006).
- It was found that α-synuclein mutants associated with PD exhibit reduced transport in neurons, as shown in rat primary neuronal cortical cultures transfected with wild-type (WT), A53T or A30P α-synuclein. For instance, the rate of transport (expressed in μm/hr) was reduced of ~55% and ~60% after 3-4 hr for A30P and A53T respectively (vs Ctr-WT) (Saha et al., 2004).
- Damaged cytoskeletal proteins disrupt also mitochondrial trafficking. Mitochondria use cytoskeletal proteins as tracks for their directional movement (Nogales, 2000). The cytoskeletal system regulates not only mitochondrial movement but also their morphology and function. Therefore, damage to microtubules perturbs transport of mitochondria through axons, increasing their retrograde movement. These changes in mitochondria dynamics lead to a decrease of mitochondria numbers in axons and mitochondria accumulation in cell bodies (De vos et al., 2007; Miller and Sheetz, 2004). Depletion of mitochondria quantity and function in axons occurs in neurodegenerative disorders (Brownlees et al., 2002; Stamer et al., 2002). Since mitochondria are ATP suppliers and microtubules need ATP to accomplish their function, mitochondrial dysfunction has a profound effect on axonal transport and function (De Vos et al., 2008).
- Mitochondrial dysfunction may damage mitochondrial trafficking through calcium dysregulation. Cytosolic Ca2+ is one of the best-studied regulators of mitochondrial movement. Elevation of cytosolic Ca2+ stops both the anterograde and retrograde trafficking of mitochondria in neurons and in many cell lines. (Chang et al. 2006; Szabadkai et al. 2006). In H9c2 cells simultaneous measurements of free Ca2+ levels and mitochondrial dynamics showed that 50% reductions in mitochondrial movement occurred at concentrations of approximately 400 nM Ca2+, and a complete arrest in the low micromolar range (Yi et al. 2004; Saotome et al., 2008). These are indirect proofs suggesting that inhibition of CI, followed by mitochondrial dysfunction, could damage mitochondrial trafficking. Also, chronic exposure to rotenone (50 nM at different times of exposure) was reported to reduce mitochondrial movement in differentiated SH-SY5Y cells (e.g., ~30% reduction of mitochondrial movement (μm/sec) after 8 days of rotenone treatment vs Ctr) (Borland et al., 2008).

Human studies

- In PD patient postmortem cortical tissues, levels of oligomeric α-synuclein in SNpc (~1000% vs Ctr samples) and expression of LC3-II levels (~130% vs Ctr samples) were up-regulated (Yu et al., 2009) (for further info, see the review from Vekrellis et al., 2011).
- The pathological observations in PD autopsy brains showed that LC3-II levels were elevated in the SNpc

and amygdala of PD brain samples, suggesting an increase in macroautophagy (but they did not reach statistical significance). LC3 colocalized with α-synuclein in most LBs and Lewy neurites in PD SNpc as well as in small punctate α-synuclein immunoreactive inclusions (IC images) (Alvarez-Erviti et al., 2010).

- Analogously, another study reported that brain homogenates derived from the temporal cortex of dementia with LB (DLB) patients vs non-demented controls were characterized by higher levels of both mTor (~130% vs Ctr) and p-mTor (~ 10 folds higher than Ctr), and levels of Atg7 (molecular initiator of autophagy) were moderately reduced in DLB cases compared to Ctr (~ 40% lower than Ctr). Consistent with the studies in human brains, levels of both mTor and p-mTor were increased in the membrane fractions from brains of α-synuclein tg mice compared to non tg controls (respectively, by ~250% and ~200% vs Ctr), and levels of Atg7 were reduced in α-synuclein tg brains compared to non tg controls (~75% less than Ctr) (Crews et al., 2010).
- Another study showed that post-mortem brain samples derived from PD patients, compared to age-matched controls, presented significant reductions of LAMP1 (2069.10 ± 329.52), CatD (1809.35 ± 533.47), HSP73 (2604.92 ± 494.56), and 20S proteasome (1660.84 ± 229.87) calculated by optic density (OD) measures (Chu et al., 2009). These data globally indicate that the functions of both the UPS and ALP systems is compromised in PD patients.

Uncertainties or Inconsistencies

- The exact molecular link from mitochondrial dysfunction to disturbed proteostasis is not known. It is not clear which is the oxidative modification that drives the process.
- The sequence of events taking place after inhibition of CI is not entirely clear (Zaltieri et al., 2015). Some studies suggest that induced oxidative stress leads to α-synuclein aggregation that triggers proteosomal dysfunction (Betarbet et al., 2006). Such order of events is suggested to take place in vivo (McNaught and Jenner, 2001). However, in other studies opposite sequence of events is proposed suggesting that first proteosomal dysfunction take place that leads to α-synuclein aggregation.

A vicious circle is observed here as α-synuclein aggregation potentiates proteosomal dysfunction and v/v. In this vicious cycle it is difficult to establish exact quantitative relationship of these two events.

- Whether α-synuclein is a substrate for proteasome remains controversial since both positive and negative data have been reported (Paxinou et al., 2001). Furthermore, polyubiquitination of α-synuclein, a prerequisite for 26S proteasomal degradation has yet to be reported (Stefanis et al., 2001). It is also not clear whether polyubiquitination of α-synuclein is necessary for its degradation. However, α-synuclein gets targeted by the UPS in the SHSY5Y neuroblastoma cell line. Phosphorylated α-synuclein gets targeted to mono- or di-ubiquitination in synucleinopathy brains (Hasegawa et al., 2002), but it is not clear if this modification can play any role in proteasomal degradation since monoubiquitination of proteins serves mainly as a signal for endocytosis or membrane trafficking.
- On the contrary to the increased α-synuclein levels observed in the midbrain, decreased α-synuclein levels were found in the cerebellums of PD patients when compared to controls, suggesting an imbalance of α-synuclein levels in different parts of the brain (Westerlund et al., 2008).
- Although mitochondrial alterations have been reported in PD patients (Ikawa et al., 2011) and disease models, it is not clear whether they represent a primary pathogenic mechanism. In particular, the critical interplay between mitochondrial dysfunction and oxidative stress, which has been widely reported in PD (Dias et al., 2013) and could constitute either a cause or a consequence of mitochondrial damage, hampers an effective comprehension of the above mentioned studies. Oxidative stress can constitute a bridge connecting mitochondrial dysfunction to the induction of α-synuclein misfolding, aggregation, and accumulation, but otherwise it may be also triggered by these latter events that in turn could induce mitochondrial alterations (Zhu and Chu, 2010; Dias et al., 2013).
- It is still unclear whether the involvement of α-synuclein in chronic MPTP toxicity reflects a physiological function for α-synuclein that has been activated in the wrong context, or whether α-synuclein produces an accidental pathogenicity that contributes to MPTP toxicity but is unrelated to the normal function of α-synuclein (Fornai et al., 2005).
- The inconsistent effects of MPP+ on autophagy (up or down regulation) are reported. It may be attributed to differences observed between immortalized cell lines and primary neurons, different timing or dose. While dysregulation of autophagy is always described, the direction is not clear. Further studies are required to clarify this issue.
- MPTP administration does not induce Lewy body formation (in contrast to rotenone) characteristic of PD, even after repeated injections (Drolet et al., 2004; Dauer et al., 2002).
- There is also controversy over whether the increase in autophagic markers is protective or, on the contrary, causative of neuronal death.
- MPP+ may have effects apart from CI inhibition, e.g., on microtubules but it is still unclear whether this is a primary effect. Indeed, MPP+ binds to microtubules in PC12 cells and inhibits their polymerization and stability (Cappelletti et al., 1999; Cappelletti et al., 2001).
- It is not clear whether microtubules disruption may be associated with α-synuclein aggregation since

tubulin was shown to co-localize with α-synuclein in Lewy bodies. Furthermore, tubulin folding is dependent on ATP and GTP hydrolysis, and mitochondrial dysfunction with subsequent energy failure could trigger microtubules disruption. Cytoskeletal microtubule (MT) injury is likely to be responsible for altered rearrangement and movement of cell organelles, being a common feature of several neurodegenerative diseases including PD (Wade, 2009; Mattson et al., 1999).

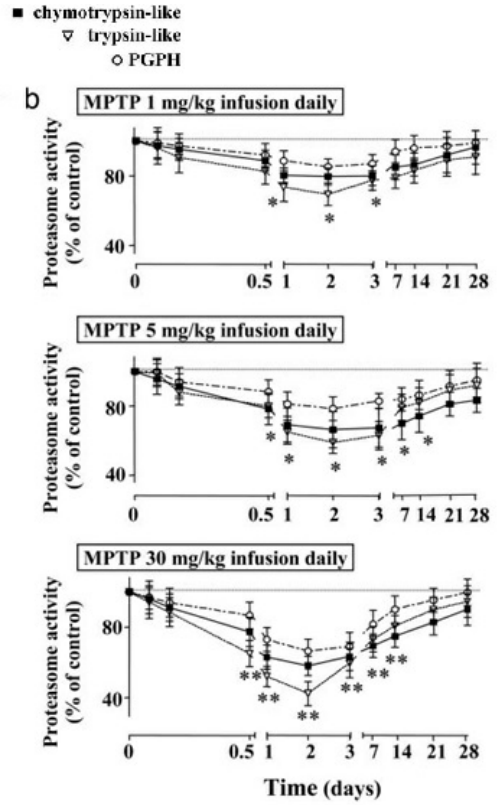
- It is not clear whether rotenone could cause microtubules depolymerization in vivo and in vitro (Brinkley et al., 1974) by binding to the colchicine site on tubulin heterodimers (Marshall et al., 1978). Ren and Feng (2007) found that microtubule depolymerization induced by rotenone caused vesicle accumulation in the soma and kills neurons.

Quantitative Understanding of the Linkage

As described in the studies above (Empirical support for linkage) a quantitative or semi-quantitative relationship has been established between rotenone-induced mitochondrial dysfunction and the impairment of UPS/ALP function. Below some representative studies are reported as examples for how such quantitative evaluations can be performed.

- Human neuroblastoma SK-N-MC or human embryonic kidney (HEK) cells were exposed to rotenone at 100 nM for 24 or 48 hrs (for further details see Chou et al., 2010).
- PD patient-derived fibroblasts (vs Ctr fibroblasts) treated with rotenone (20 and 500 μM for 6 h for the evaluation of protein quality control system or 100 nM, 1 μM and 10 μM for 1 h for redox experiments) showed reduction of UPS function (as shown by higher induction of 20S proteasome activity in PD fibroblasts vs Ctr after both 20 and 500 μM rotenone administration). An increase of LC3-II accumulation in both groups (PD and Ctr) after exposure to 500 μM rotenone was observed suggesting that (Ambrosi et al. 2014).
- Human neuroblastoma cells (SK-N-MC) after short treatment with rotenone (1 week) elevated soluble α-synuclein protein (41 ± 16% increase) levels without changing mRNA levels, suggesting impairment of α-synuclein degradation via UPS. Chronic rotenone exposure (4 weeks) increased levels of insoluble α-synuclein (29 ± 9% increase) and ubiquitin (87 ± 14% increase) (Sherer et al., 2012).
- SHSY-5Y cells treated with rotenone (500 nM, 24 h) showed a ~2 fold increase in DCF fluorescence compared to untreated cells (indicative of intracellular ROS). Additionally, rotenone elevated cytosolic calcium (about 35-40% increase vs Ctr), ER-stress (about 45% increase vs Ctr), impaired UPS function (~3 fold increase of insoluble protein aggregate vs Ctr). Inhibition of Rac1 (Rho-like GTPase) mitigated the oxidative/nitrosative stress, prevented calcium-dependent ER-stress, and partially rescued UPS function (Pal et al. 2014).
- Human neuronal SH-SY5Y cells treated with rotenone (10 μM, for 24 hr showed accumulation of high molecular weight ubiquitinated bands (by immunoblotting – qualitative - assay), and increase of both mitochondrial- (~5 fold increase vs Ctr) and cytosolic- cytochrome c fractions (~1.2 fold increase vs Ctr). Rapamycin pre-treatment (3 μM, for 48 hr prior addition of rotenone) diminished rotenone-induced effects, as shown by enhanced degradation of ubiquitinated proteins, and reduced levels of cytosolic cytochrome c. Also, rapamycin promoted mitophagy (as shown by lysosome and mitochondria co-localization within the cells) (Pan et al. 2009).

Examples of quantitative evaluation of this KER



(https://aopwiki.org/wiki/index.php/File:KER_3_Fig.1._proteosome_activity.jpg)

Fig.1. Dose and time dependent striatal proteasome activity after MPTP continuously infused upto 28 days measured by relative chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-peptide hydrolysing (PGPH) proteasome activities in mice. Delayed and prolonged inhibition of proteasome activity after continuous MPTP administration (1, 5, or 30 mg/kg MPTP daily) for the indicated time periods. Asterisks indicate statistically significant differences (P _<0.05) from baseline proteasome activity (single asterisk) or from both baseline proteasome activity and activity after lower MPTP doses (1 and 5 mg/kg, daily, double asterisk; n =5 mice) (Fornai et al., 2005, Fig. 2 B).

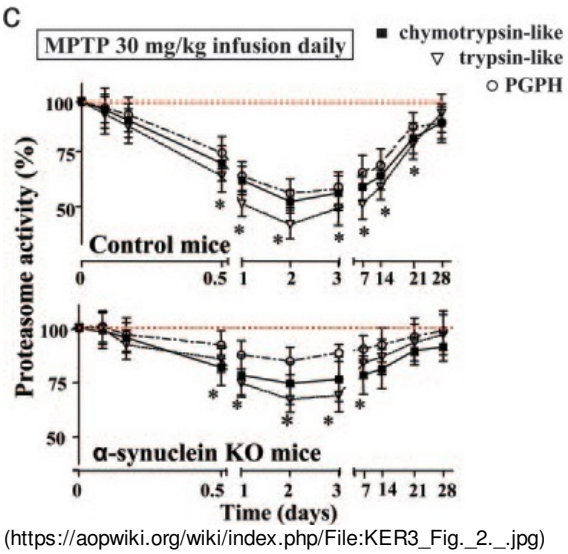


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Fig. 2. Effect of α-synuclein deletion on MPTP toxicity. Proteasome activity in control and alpha-synuclein KO mice continuously infused for 28 days with MPTP (30 mg/kg of body weight daily, striatum concentration approximately 13 uM). Proteasome activities in the substantia nigra are depicted as percent of control (means +/- SEMs) as a function of time after beginning of the infusions (five mice per group). Asterisks indicate statistically significantly different values (P < 0.05) from controls (Fornai et al., 2005).

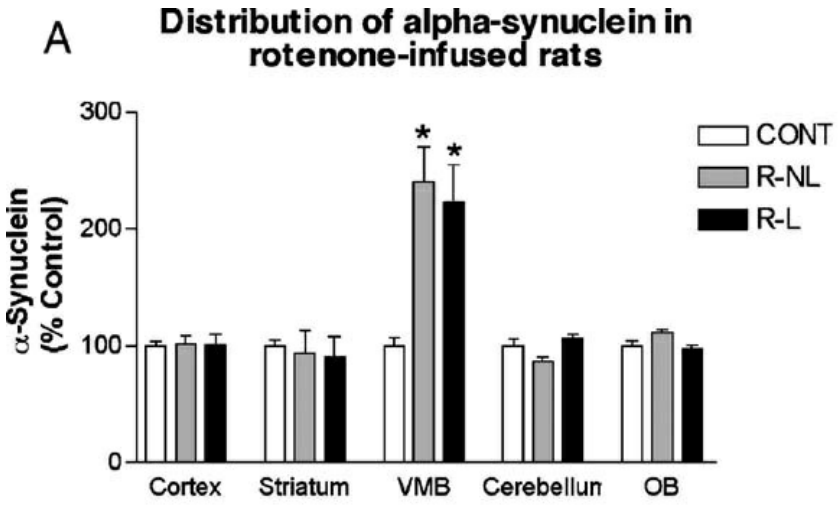

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Fig. 3. α-Synuclein levels were selectively increased in the ventral midbrain (VMB) region of rotenone-infused rats with or without lesion. α-Synuclein levels, as determined from Western blot analysis, from rotenone-treated rats were expressed as a percentage of values from control vehicle-infused rats. Results are mean ± SEM (n = 3 control, 6 rotenone with lesion, 3 rotenone with no lesion) *P < 0.05 vs. vehicle-infused rats (from Betarbet et al., 2006, Fig. 3A).

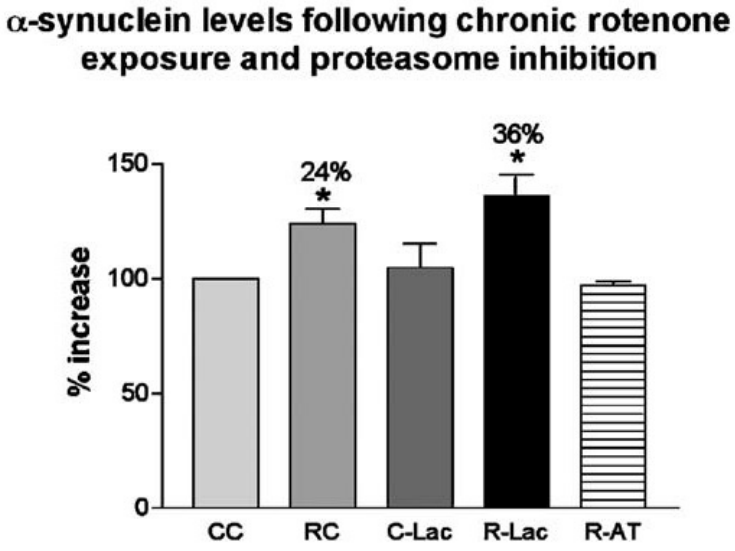

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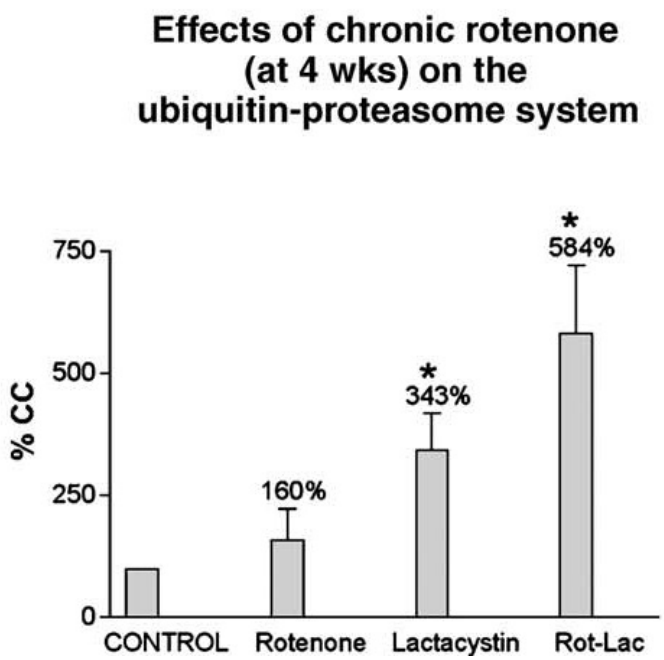
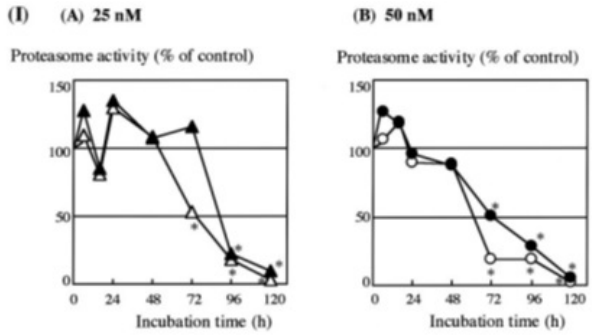
Fig. 4. Bar graph showing the effects of rotenone and lactacystin on α-synuclein levels after 4 weeks of rotenone exposure (5 nM) in vitro, on SK-N-MC human neuroblastoma cells. Rotenone alone increased α-synuclein levels, but lactacystin alone did not. α-Tocopherol attenuated the rotenone-induced increase in α-synuclein. Results are mean ± SEM (n = 4). *P < 0.05 vs. solvent-treated cells. CC, control cells; RC, rotenone-treated cells; C-Lac or CL, lactacystin treated cells; R-lac or RL, rotenone and lactacystin treated cells; R-AT, rotenone and α-tocopherol treated cells (from Betarbet et al., 2006, Fig. 5B).



(https://aopwiki.org/wiki/index.php/File:KER3_Fig._5.png)

Fig.5. Levels of ubiquitinated proteins were estimated in solubilized protein fractions from SK-N-MC cells collected at the end of each week of rotenone treatment (5 nM), using gel electrophoresis and immunoblotting. Quantitative analysis demonstrated significant increases in ubiquitinated protein levels 4 weeks after rotenone treatment and after proteasomal inhibition with lactacystin. Band intensities were expressed as % of control. Results represent mean ± SEM. *P < 0.05 compared to control (from Betarbet et al., 2006, Fig. 8C).



(https://aopwiki.org/wiki/index.php/File:Fig._6.jpg)

Fig. 6. Effects of rotenone on the activity of proteasome. Proteasome activity in the cytoplasmic fraction of cells treated with 25 nM (A) or 50 nM (B) rotenone was measured fluorometrically in the absence (open triangles and circles) or presence (solid triangles and circles) of exogenously added ATP (2 mM) (from Shamoto-Nagai et al., 2003, Fig. 6).

KE (upstream) Mitochondrial dysfunction	**KE3 (downstream)** **Impaired proteostasis** UPS inhibition (% approx.) measured by:		**Comments**	**References**
Rotenone (nM) **(in vitro)**	**26S UPS activity**	**+ catalase** **(anti-oxidant)**	HEK cells exposed for 2 4hr	**Chou et al., 2010**
10	24	Not done		
100	48	Increased UPS activity by 40%		
1000	60	Not done		

	20S proteasome activity	SK-N-MC human neuronal cell line (exposed for 24 hr)	**Chou et al., 2010**
1	8		
50	4		
100	18		
500	22		
1000	24		
	20S proteasome immune-reactivity decrease		
10	22		
100	48		
100	70		
MPTP **(in vivo)**	**Chymotrypsin-like UPS activities (at day 2)**		
1 mg/kg daily	20	Mice continuously infused with MPTP for 28 days	**Fornai et al., 2005**
5 mg/kg daily	30		
30 mg/kg daily	40		
	Trypsin-like UPS activities (at day 2)		
1 mg/kg daily	30		
5 mg/kg daily	40		
30 mg/kg daily	60		
	Peptidyl-glutamyl-peptide hydrolysing (PGPH) UPS activities (at day 2)		
1 mg/kg daily	20		
5 mg/kg daily	20		
30 mg/kg daily	30		

Table. 1. These studies showed that rotenone caused a reduction in UPS activity (measured by 26S and 20S proteasome activity) in a dose-dependent manner. Further studies showed that rotenone increases proteasome subunit degradation, but does not alter synthesis (Western blot and RT-PCR studies, reviewed in Chou et al., 2010). Dose- and time- dependent striatal proteasome activity is also shown after MPTP continuously infused up to 28 days measured by relative chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-peptide hydrolysing (PGPH) proteasome activities in mice (Fornai et al. 2005).

- PD patient-derived fibroblasts (vs Ctr fibroblasts) showed reduction of UPS function (by ~33%) and higher accumulation of ubiquitinated proteins (by ~2 fold) in PD as compared to control fibroblasts at baseline. Treatment with rotenone (20, 500 μM, 6hr) caused a higher induction of 20S proteasome activity in PD fibroblasts vs Ctr. An increase of LC3-II accumulation (indicative of autophagic vesicle accumulation) in both groups (PD and Ctr) after exposure to 500 μM rotenone was observed (Ambrosi et al. 2014).
- Human neuroblastoma cells (SK-N-MC) after short treatment with rotenone (1 week) elevated soluble α-synuclein protein (41 ± 16% increase) levels without changing mRNA levels, suggesting impairment of α-synuclein degradation via UPS. Chronic rotenone exposure (4 weeks) increased levels of insoluble α-synuclein (29 ± 9% increase) and ubiquitin (87 ± 14% increase) (Sherer et al., 2012).
- SHSY-5Y cells treated with rotenone (500 nM, 24 h) showed a ~2 fold increase in DCF fluorescence compared to untreated cells (indicative of intracellular ROS). Additionally, rotenone elevated cytosolic calcium (about 35-40% increase vs Ctr), ER-stress (about 45% increase vs Ctr), impaired UPS function (~3 fold increase of insoluble protein aggregate vs Ctr). Inhibition of Rac1 (Rho-like GTPase) mitigated the oxidative/nitrosative stress, prevented calcium-dependent ER-stress, and partially rescued UPS function (Pal et al. 2014).
- Human neuronal SH-SY5Y cells treated with rotenone (10 μM, for 24 hr showed accumulation of high molecular weight ubiquitinated bands (by immunoblotting – qualitative - assay), and increase of both

mitochondrial- (~5 fold increase vs Ctr) and cytosolic- cytochrome c fractions (~1.2 fold increase vs Ctr). Rapamycin pre-treatment (3 μM, for 48 hr prior addition of rotenone) diminished rotenone-induced effects, as shown by enhanced degradation of ubiquitinated proteins, and reduced levels of cytosolic cytochrome c. Also, rapamycin promoted mitophagy (as shown by lysosome and mitochondria co-localization within the cells) (Pan et al. 2009).

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impaired, Proteostasis leads to Degeneration of dopaminergic neurons of the nigrostriatal pathway (https://aopwiki.org/relationships/905)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Strong**	**Moderate**

Evidence Supporting Applicability of this Relationship

Multiple animal modeles have been used to mimic PD (Johnson et al. 2015). There are no sex restriction; however, susceptibility to MPTP increases with age in both non-human primates and mice (Rose et al.1993, Irwin et al. 1993, Ovadia et al. 1995).

How Does This Key Event Relationship Work

One of the critical functions in the long-lived cells such as neurons is the clearing system for the removal of the unfolded proteins. This function is provided by two major systems, the Ubiquitin Proteosome System (UPS) and the Autophagy-Lysosome Pathway (ALP) (Tai HC et al. 2008; Korolchuck VI et al. 2010 and Ravikumar B et al. 2010). Impaired proteostasis with formation of misfolded α-synuclein aggregates deregulates microtubule assembly and stability with reduction in axonal transport and impairment of mithocondrial trafficking and energy supply (Esposito et al. 2007; Chen et al. 2007; Borland et al. 2008; O’Malley 2010; Fujita et al. 2014; Weihofen et al. 2009).

Pathological consequences of these deregulated process include interference with the function of synapses, formation of toxic aggregates of proteins, impaired energy metabolism and turnover of mitochondria and chronic endoplasmic reticulum stress; all eventually leading to degeneration of DA neurons in the nigrostriatal pathway (Fujita et al. 2010, Shulman et al. 2011, Dauer et al. 2003, Orimo et al.2008, Raff et al. 2005; Schwarz 2015).

Weight of Evidence

The weight of evidence for the relationship between impaired proteostasis and degeneration of dopaminergic neurons of the nigrostriatal pathway is strong. The biological plausibility is based on the knowledge of the physiological cellular process governing the cleaning processes of degradated proteins and organells and on the observations done in genetic and idiopathic forms of Parkinson's disease. Dose and time concordance support a strong response-respose relationships which is also supported by the very well known chronic and progressive behviour of the Parkinson's disease. Although essentiality has been demonstrated in multiple models and lines of evidence, including knockout animals, a single molecular chain of events cannot be established; therefore essentiality for this KEs relationship was considered moderate.

Biological Plausibility

The fact that impaired proteostasis can induce degeneration of DA neurons of the nigrostriatal pathway is well known and based on the understanding of the physiological cellular processes involved in removing degraded/misfolded proteins as they are critical for normal mitochondria and axonal transport. Accumulation of misfolded and/or aggregated α-synuclein and the presence of abnormal mitochondria is a consequence of deregulation of this clearing process, and the Lewy bodies, a pathological hallmark of sporadic PD, stain specifically for proteins associated with UPS (Fornai et al., 2003; Gai et al., 2000; McNaught et al., 2002).

Impaired proteostasis has been described in humans affected by sporadic PD (McNaught et al.; 2001, 2003), and changes induced by excess cellular levels of degraded proteins in nigral dopaminergic neurons cause a progressive decline in lysosome function, i.e. ALP system, contributing to neurodegeneration (Decressac et al. 2013). In this context, the ALP system is likely working in a complementary way, with the UPS being the major cleaning system in the soma and the ALP playing a role at pre-synaptic sites (Friedman et al., 2012).

Pathological observations from patients affected by PD and from animal models show an increased number of autophagic vacuoles or autophagic markers (Alvarez-Erviti et al., 2010; Crews et al. 2010). Additional observations support the role of impaired proteostasis in nigrostriatal toxicity such as : several genetic variants of sporadic PD are due to susceptible genes able to participate in or modify proteostasis (Shulman et al. 201, Fornai et al. 2003, Shimura et al. 2000, Leroy et al.1998) and striatal microinfusion of proteasome inhibitors induce selective nigrostriatal toxicity with loss of DA and DA metabolites (DA, DOPAC and HVA) in the striatum, retrograde loss of nigral DA cell and intracytoplasmatic inclusions positive for protein of the UPS (Fornai et al. 2003).

Transgenic overexpression of mutant or wild-type forms of α-synuclein in mice causes neuropathological changes including dystrophic neurites and α-synuclein positive LB-inclusion (Dauer et al. 2003; Masiliah et al. 2000). However, they fail to reproduce specific cell death in the nigrostriatal pathway. In contrast, injection of human α-synuclein expressing viral vectors into the SN of adult rats causes a selective death of dopaminergic neurons and formation of LB inclusions (Dauer et al. 2003; Kirik et al. 2002; Lo Bianco et al. 2002). These effects were observed with adeno-associated virus –mediated expression of A30P α-synuclein and with lentiviral-mediated expression of α-synuclein in rats, mice and non-human primates (Shulman 2010; Kirk et al.2003; Klein et al. 2002; Lo Bianco et al. 2002 and 2004; Lauwerset al. 2003).

Impaired proteostasis and formation of proteins aggregates also affect the axonal transport and mitochondrial trafficking. α-synuclein mutants accumulate in the neuronal soma when overexpressed, reducing the axonal transport (Kim-Han et al. 2011; Saha et al.2004); in addition, overexpressed vesicle-associated α-synuclein binds to the microtubules with a detrimental role on axonal transport (Kim-Han et al. 2011; Yang et al. 2010). Postmortem studies on PD patients are indicative of axonal damage. It appears that axonal changes precede neuronal loss, supporting the idea that axonal impairments are early events in neurodegenerative disorders (Orimo et al. 2005 and 2008, Raff 2002, Braak et al. 2004). These changes, and observation from animals models using the chemical stressor MPTP (Meissner et al. 2003, Serra et al. 2002, Hasbani et al. 2006) are supporting the notion that DA neurons of the nigrostriatal pathway degenerate through a “dying back” axonopathy (Raff et al. 2002). It was demonstrated that axonal degeneration follows an active process distinct from cell body loss in a Wallerian degeneration slow (WldS) mutant mouse transgenic model. In this model, axonal degeneration in a variety of disorders is inhibited. In WldS mice, acute treatment with MPTP (20 mg/kg ip for 7 days) resulted in attenuated nigrostriatal axon degeneration, and attenuated DA loss, but cell bodies were not rescued (Hasbani et al. 2006). Indeed, multiple evidences from genetic and experimental models (particularly using MPTP as a chemical stressor) support an early and critical role of axonal impairment with early occurrence of Lewy neurites preceding Lewy bodies formation and cell death (O’Malley 2010).

In addition, a strong link between mitochondrial dysfunction and PD came from the discovery that mutations in PINK1, α-synuclein, LRRK2, parkin and DJ-1, all linked with genetic causes of PD, can affect mitochondrial function (Rappold et al.2014, O’Malley 2010). Deregulation of mitochondrial dynamics (fission, fusion and movement of mitochondria) can affect neuronal activity and viability and imbalance of mitochondrial dynamics have been reported in experimental models of PD with mutated α-synuclein (Tieu, 2014) or chronic model of primary neuronal cells treated with low concentrations (0.1-1 nM) of rotenone (Arnold et al. 2011). Progression of neuronal changes with formation of Lewy neurites and reduction of mitochondrial movement leading to cell death has been also observed in-vitro in a chronic cell-based model (SH-SY5Y neuroblastoma cell line) treated with Rotenone (50nM for 21 days). In this assay, reduction in mitochondrial movement was associated with a progressive damage, first including formation of Lewy neurites, followed by cell death (Borland et al.2008).

Empirical Support for Linkage

Degeneration of DA neurons of the nigrostriatal pathway, similar to the one observed in PD, have been reproduced in human and experimental animal models following exposure to MPTP (Dauer 2003; Kitamura et al. 2000; Meissner et al. 2003; Serra et al. 2002; Langston et al. 1983; Rose et al. 1993; Irwin et al. 1993; Forno et al. 1993; Ovadia et al. 1995; Porras et al. 2012) and in animals following administration of rotenone through multiple routes of exposure (Betarbet et al. 2000 and 2006, Fleming et al.2004, Schmidt et al. 2002, Inden et al.2007, Saravanan et al. 2005, Sherer et al. 2003; Pan-Montojo et al. 2010 and Johnson et al. 2015). This indicates that both chemicals can be used as a tool compound for experimental investigations on PD and exploring the key event relationship between impaired proteostasis and degeneration of DA neurons of nigrostriatal pathway. Also, similar to PD, susceptibility to MPTP increases with age in both non-human primates and mice (Rose et al.1993, Irwin et al. 1993, Ovadia et al. 1995). It should be noted that the upstream key event includes multiple pathological events, eventually leading to the downstream key event. As it is difficult to assess real time changes for a series of complex and dynamic events in a single experiment, most of the empirical supporting evidences are performed by exploring single factors (e.g. impairment of ALP or UPS or axonal transports) and their role in the degeneration of DA neurons. A selection of studies supporting the empirical evidence is reported below.

Empirical support using MPTP/MPP+

- Inhibition of the UPS was observed following continuous infusion of MPTP at 1, mg/kg/day for 28 days in mice. A dose related decrease in the enzyme activity of the UPS was observed and this effect was associated with a dose-related decrease of TH positive terminals (densitometry analysis) in the dorsal and ventral striatum. This effect was accompanied by a dose-related cell loss in the SN (counting of TH positive cells) at 5 and 30 mg/kg/day. At 30 mg/kg/day the authors reported cytoplasmic inclusions positively staining for ubiquitin and α-synuclein in neurons of the SN (and locus coeruleus). In the same

experiment, acute administration of MPTP (single injection of 30 mg/kg/ or 4 separate injections of 20 mg/kg) induced a transient inhibition of the UPS activity, neuronal loss but no intracytoplasmatic inclusions, indicating that a continuous infusion is necessary to induce permanent inhibition and pathological changes similar to the one observed in PD (Fornai et al. 2005).

- In mice lacking α-synuclein, continuous infusion of up to 30 mg/kg/day for 28 days of MPTP neuronal cell death and behavioral symptoms were almost alleviated (Fornai et al. 2005, Dauer et al. 2002).
- Administration of MPTP to mice (30 mg/kg/day ip for 5 days) produced autophagosome (AP) accumulation (increase in LC3II) and dopaminergic cell death which was preceded by a decrease in the amount of lysosomes in DA neurons. MPTP also induced mitochondrial- derived ROS and permeabilization of the lysosomal membrane. This resulted in a decrease in Lamp 1 lysosome structural protein and accumulation of undegraded AP and release of lysosomal enzymes into the cytosol. The effect observed in-vivo was quantitatively confirmed in-vitro (human neuroblastoma cell line BEM17(M17EV)). MPP+ was tested in-vitro at the concentrations of 0.25 to 2.5 μM and induced a concentration- related decrease in Lamp1, increase in LC3II, increase in cell death and decrease in lysotracker. In the same in-vitro system, MPP+ also induced lysosome membrane permeabilization. In the same experiment, induction of lysosome biogenesis by the autophagy-enhancer compound rapamycin attenuated the dopaminergic neurodegeneration, both in vitro and in vivo, by restoring lysosomal levels (Dehay et al. 2010).
- In an in-vitro microchamber that allowed specific exposure of neuritis of murine mesencephalic neurons, treatment with 1 to 5 μM of MPP+ induced impairment of mitochondrial transport, neurite degeneration (degeneration of proximal dendrites) and autophagy, before cell death (Kim-Han et al. 2011). The number of TH positive cell bodies and neurites was reduced at 1 μM, and axonal fragmentation and LC3 dots increased while tubulin density decreased (Kim-Han et al. 2011).
- Mice treated with MPTP at 20mg/kg/day ip for 5 days showed loss of DA neurons in SN which was attenuated by the pharmacological block of mitochondrial fission protein Drp1. Drp 1 blockade also promoted mitochondrial fusion and enhanced the release of DA from the striatal terminals in a PINK1 knockout model showing a defective DA release (Rappold et al. 2014; Tieu et al. 2014).
- In differentiated (d6) LUHMENS cell system stably expressing eGFP/mito-tRFP, treatement with MPP+ (5μM) for 24 hours revealed a reuction in the total number of mitochondria in neuritis and a significant reduction in velocity. Partial protection from MPP+ dependent mitochondrial immobilization in neuritis as well as from drop in mitochondria numbers in neuritis was detects following co-treatment with the anti-oxidant Vitamin C (Schildknecht et al. 2013)

Proteasome inhibitors

- Intracerebral microinfusion of proteasome inhibitors (lactacystein or epoxomycin at , 100 and 1000 μM) induced loss of TH and DAT immunostaining and decrease in DA and DOPAC in DA terminals in the striatum and loss of nigral cells in SN (counting of TH positive cells). Formation of cell inclusions (positively immunostained for α-synuclein and ubiquitin) and apoptosis were observed after treatment with proteasome inhibitors (0.1 to 50 μM) in an in-vitro system (PC 12 cells). The concentration response curve for apoptosis was shifted to the right compared to the concentration response curve for cellular inclusions indicating that inclusions occurred earlier and independently of cell death. A maximum effect was reached between 1 and 10 μM (Fornai et al.2003).

Empirical support using Rotenone

- Administration of rotenone, via osmotic mini pumps implanted to rats (3 mg/kg/day for 7 days) induced decrease of TH in substantia nigra and striatum and decrease in α-synuclein, in its native form, in substantia nigra and striatum, while monoubiquitinated alpha-synuclein increased in the same regions. Valproic acid (VPA) treatment (effective inhibitor of histone deacetylases) significantly counteracted the death of nigral neurons and the 50% drop of striatal dopamine levels caused by rotenone administration.VPA treatment also counteracted both type of α-synuclein alterations. Furthermore, monoubiquitinated alpha-synuclein increased its localization in nuclei isolated from substantia nigra of rotenone-treated rats, an effect also prevented by VPA treatment. Nuclear localization of alpha-synuclein has been recently described in some models of PD and its neurodegenerative effect has been ascribed to histone acetylation inhibition (Monti et al. 2010).
- Chronic oral administration of rotenone at 30mg/kg/day in mice produced neuronal loss and degeneration of TH positive terminals in the striatum accompanied by an increase in α-synuclein, ubiquinated proteins and decrease in proteaosomal activity. Concomitant treatment with 4-PBA (a chemical chaperone able to reverse the mislocalization and/or aggregation of proteins) inhibited rotenone-induced neuronal death and decreased protein level of α-synuclein (Inden et al. 2007).
- Treatment of Lewis rat with 2 mg/kg/day of rotenone, administered sc for 8 weeks impaired autophagic flux, induced lysosomal dysfunction and degeneration of DA neurons (decrease in number of TH positive cells and decrease in density of TH positive fibers) in SNpc . The effect of rotenone was paralleled by an increase in LC3 immunopositive dots and upregulation of the LC3II in DA neurons. A concomitant effect was observed and characterized by a decrease in LAMP2 and catepsin immunodots with a diffuse morphological pattern, possibly indicative of decreased lysosomal membrane integrity and leaking to cytosol. In-vitro (PC12 cells) at 500 nM, rotenone also induced increases in α-synuclein, microtubule associated protein 1, light chain 3-II, Beclin 1, p62, increased lysosome permeability and induced cell death. In PC12 cell, the concomitant treatment with trehalose (autophagic inducer) attenuated the

rotenone-induced cell death while in-vivo trehalose treatment decreased the rotenone-induced dopaminergic neurons loss (Wu et al. 2015).

- Rotenone LD50 of 10 nM in differentiated SH-SY5Y cells decreased autophagic flux at both 2 and 24h. Up-regulation of autophagy by rapamycin protected against cell death while inhibition of autophagy by 3-methyladenine exacerbated cell death (Giordano et al. 2014)
- Treatment of embryonic midbrain neuronal cells with 0.1 to 10 µM rotenone for 30 minutes induces a decrease in polymerized tubulin and increased the number of apoptotic TH+ cells. Similar effects were observed with colchicine treatment, a well-known microtubule-depolyrizing agent and prevented by taxol, a well-known microtubule –stabilizing agent. The effect was considered specific to DA neurons as the effect on apoptosis and cell death was much less evident in GABAergic and glutamatergic neurons (Ren et al. 2005).

Human studies

- Inclusion bodies in DA neurons (ie Lewy bodies), a pathological hallmark for sporadic PD, stains specifically for proteins associated with the UPS (Fornai et al. 2003, Gai et al. 2000, Mcnaught et al. 2002), including α-synuclein, parkin and ubiquitin; possibly indicating that failure of the UP system represents a common step in the pathogenesis of PD and impairment of the proteasome system was found in humans affected by sporadic PD (McNaught et al. 2001, 2003).
- Lysosomal breakdown and autophagosome (AP) accumulation with co-localization of lysosomal markers in Lewy Bodies is reported to occur in PD brain samples where Lewy bodies were strongly immunoreactive for the autophagosome markers (LC3II), (Dehay et al. 2010).
- Postmortem studies on PD patients show axonal pathology that is likely to precede the loss of neuronal bodies In this investigation, TH immunoreactive fibers had almost entirely disappeared with preservation of neuronal bodies (Orimo et al. 2005 and 2008).

Uncertainties or Inconsistencies

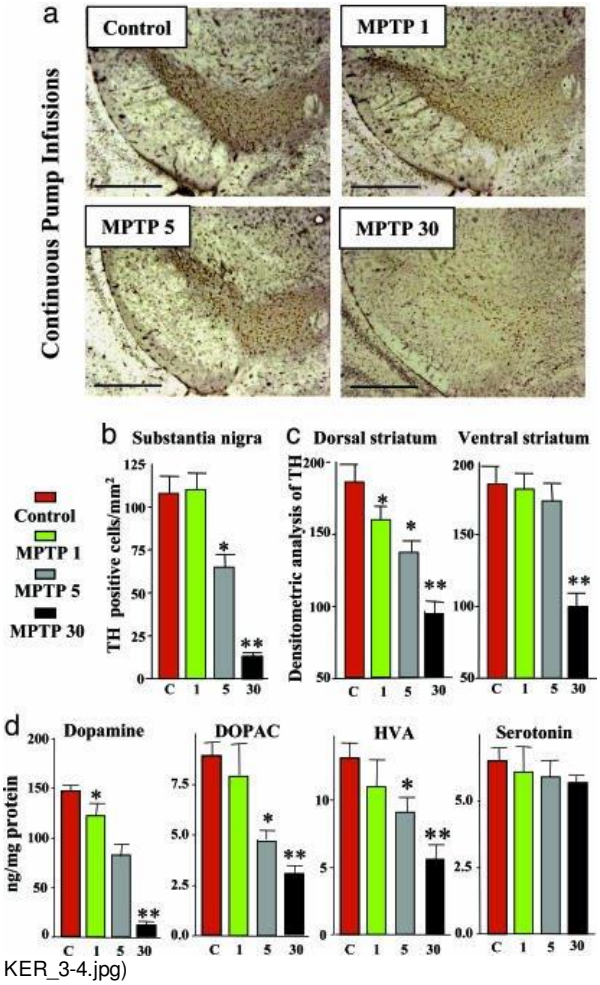
- MPTP can induce damage to nigrostriatal neurons without formation of Lewy bodies (hall mark of PD). Acutely intoxicated humans and primates with MPTP lack LB-like formation (Dauer et al. 2003; Forno et al. 1986, 1993). Similarly, discontinuous administration of rotenone, even at high doses, damages the basal ganglia but produce no inclusions (Heikkila et al. 1985; Ferrante et al. 1997, Lapontine 2004). To reproduce the formation of neuronal inclusions, continuous infusion of MPTP or rotenone is necessary.
- Acute intoxication with rotenone seems to spare dopaminergic neurons (Dauer et al 2003, Ferrante 1997). In addition, in rats chronically infused with rotenone showed a reduction in striatal DARPP-32-positive, cholinergic and NADPH diaphorase-positive neurons (Hoglinger et al. 2003) or in other brain regions. These results would suggest that Rotenone can induce a more widespread neurotoxicity (Aguilar et al. 2015).
- The vulnerability of the dopaminergic pathway still remains circumstantial. The selectivity of MPP+ for dopaminergic neurons is due to its selective uptake via dopamine transporter (DAT), which terminates the synaptic actions of dopamine (Javitch et al. 1985, Pifl et al. 1993, Gainetdinov et al.1997, Hirata et al. 2008). Selectivity of rotenone for dopaminergic neurons is not fully understood (Hirata 2008).
- Transgenic overexpression of α-synuclein induces neurotoxicity (ie neuronal atrophy, distrophic neuritis, astrocytosis and LB-like formation). However they fail to cause death of dopaminergic neurons. Nevertheless, injection of the human protein or mutated form expressing viral vectors into the SN, are able to induce all the pathological changes characteristic of PD. This discrepancy could be due to the higher expression of α-synuclein in the viral vector model or because in these models, α-synuclein overexpression would occur suddenly in adult animals (Dauer et al. 2003). In addition, transgenic expression of C-terminal truncated α-synuclein also leads to motor symptoms but neuronal degeneration is not reported (Halls et al. 2015).
- There is conflicting literature on whether increased autophagy would be protective or enhances damage. Similarly, a conflicting literature exists on extent of inhibition or activation of different protein degradation system in PD and a clear threshold of onset is unknown (Fornai et al. 2005).
- Several mechanisms may affect the axonal transport in neurons showing swelling of neurites positive for α-synuclein. These include e.g. ROS production, lysosome and mitochondria membranes depolarization, increased permeability and microtubule depolymerization (Kim-Ham et al.2011, Borland et al.2008, Choi et al.2008). As both MPTP and rotenone could directly trigger these effects, a clear mechanistic understanding leading to cell death is difficult to identify (Aguilar et al. 2015).
- Different features of imbalanced proteostasis can trigger one another (e.g. disturbed protein degradation, pathological protein aggregation, microtubule dysfunction); and each of them can lead to cell death. Therefore, the “single” triggering event triggering axonal degeneration or neuronal death is not known. For instance, for α-synuclein aggregation, it is not clear whether this causes death because some vital function of neurons is lost, or whether some protein increases e.g. because of inhibited chaperone-mediate autophagy (Kaushik et al. 2008, Cuervo et al. 2014).
- Real-time changes in DA axons are difficult to assess, accounting for the limitation of testing models of structural or trafficking impairment in-vivo.

Quantitative Understanding of the Linkage

As described in the empirical support, a quantitative relationship has been established between chemical stressors inducing impaired proteostasis and loss of DA neurons of nigrostriatal pathway. The response-response relationship was evident in most of the studies and, where possible a relationship in dose-response could be also observed. A chronic dose regimen for the chemical stressor was necessary in most of the studies and this is confirming that a long lasting perturbation of the key event up is necessary to affect neuronal loss consistent with the presence of intracytoplasmatic inclusions. However, some inconsistency in the measurement of the endpoints relevant for impaired proteostasis were observed, probably because they also act as compensatory factors (Betarbet et al. 2006). The acute administration of MPTP (single injection of 30 mg/kg/ or 4 separate injections of 20 mg/kg) induced a transient inhibition of the UPS activity and neuronal loss but no intracytoplasmatic inclusions ie Lewy body were observed, supporting the temporal relationship among the two events (Fornai et al. 2005).

Measured endpoint relevant for the KEup (KE3)	**Measured endpoint relevant for the KEdown (KE4)**	**Model**	**Reference**
Approx. 40% inhibition of UPS	Approx. 38% decrease in TH density in dorsal striatum	MPTP 1mg/kg/day IV infusion for 28 days in mice	Fornai et al. 2005
Approx.50% inhibition of UPS	Approx. 40% decrease in number of TH positive cells/mm2 in SN and approx. 25% decrease in TH in dorsal striatum	MPTP 5mg/kg/day IV infusion for 28 days in mice	
Approx.60% inhibition of UPS	Approx. 86% decrease in number of TH positive cells/mm2 in SN and approx. 50% decrease in TH in dorsal striatum and approx. 50% in ventral striatum	MPTP 30mg/kg/day IV infusion for 28 days in mice	
Approx. 40% proteasome inhibition	Approx. 70% decrease in DA and 50% decrease in DOPAC in striatum and 30% cell loss in SN	ic infusion of lactacystin (proteasome inhibitors) in rats 100 µM	Fornai et al. 2003
Approx. 50% increase in mRNA expression for α-synuclein	Decrease in TH immunoreactivity (approx. 50%), in TH-positive nerve terminals in the striatum	Transgenic model overexpressing α-synuclein	Kirk et al. 2002
Approx.16-13% reduction in proteosomal activity	Degeneration of nigrostriatal dopaminergic neurons in 50% of animals	Chronic iv treatment (up to 5 weeks) of Lewis rat with rotenone at 2-3 mg/kg day (free brain Rotenone 20-30 nM)	Betabret et al. 2000 and 2006
Approx. 50% increase in α-synuclein	Approx. 57% reduction in TH immunoreactivity in SNpc neurons at 30 mg/kg/day Decrease in TH and DATin the striatum (approx. 30% and 70% respectively) and ventral midbrain area (approx. 60%) at 30 mg/kg/day	Oral chronic administration (28 days) of rotenone (0.25, 1, 2.5, 5, 10 or 30 mg/kg/day) to mice	Inden et al. 2007
Increase in LC3 positive dots in nigral DA neurons (approx. 380%), upregulation of LC3II (approx. 40%), Beclin 1 (approx. 33%) and P62 (approx. 50%) autophagic substrate	Approx.40% decrease in the number of TH neurons (SNpc) and density of TH positive fibers (approx.50%) (striatum).	2mg/kg/day for 8 wks sc of Rotenone in Levis rats	Wu F. et al., 2015
Approx. 8 fold increase in the number of TH+ neurons with granular LC3	Approx. 40 % decrease in the number of TH immunoreactive neurons.	Primary dopaminergic neurons following treatment with MPP+ (LD50 of 5µM/L)	Zhu et al. 2007

Decrease in mitochondrial speed (approx. 100% decrease in anterograde speed and approx. 28% increase in retrograde speed)	Approx. 70% decrease in positive TH neuronal bodies at 48hours	Treatment with up to 5 µM (1 to 5 µM) of MPP+ in TH positive murine mesencephalic neurons in an in-vitro microchamber segregating system	Kim-Ham et al. 2011
Reduction in mitochondrial movement was statistically significant from day 8 and was greatest on day 16 at 50 nM (approx. day 3 19%, day 6 7%, day 8 62%, day 14 37%, day 16 200%)	Approx 60% of cell loss by day 21	In vitro SH-SY5Y neural cells treated with 50 nM rotenone for 21 days	Borland K. et al., 2008
30% increase over control in static mitochondria and 50 decrease over control in number of mitochondria	Significant decline of intracellular ATP at 24 hours	differentiated (d6) LUHMENS stably expressing eGFP/mito-tRFP, treated with MPP+ (5µM) for 24 hours	Schildknecht S. et al. 2013



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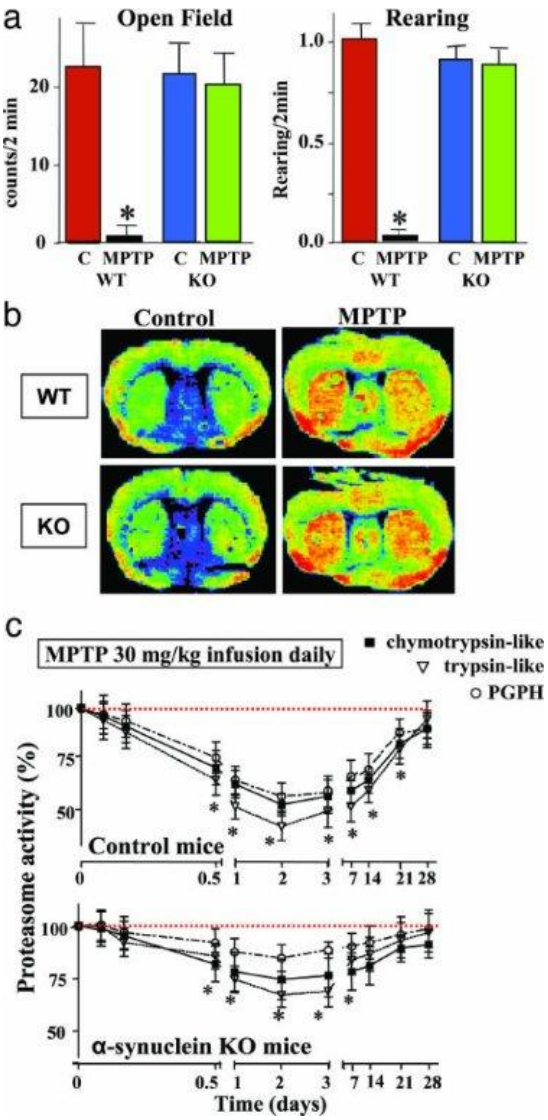
Neurotoxicity induced by continuous MPTP administration. (a) Representative tyrosine hydroxylase (TH)-stained sections of the substantia nigra from mice that were continuously treated for 28 days with control pump infusions or with infusions of 1, 5, or 30 mg MPTP/kg daily. (Scale bar, 600 µm.) (b and c) TH-positive cell counts in the substantia nigra (b) and semiquantitative densitometric measurements of the TH signal in striatum (c)(n = 10 mice per group). (d) Striatal monoamine levels in MPTP-treated mice (n = 10 mice per group). Asterisks indicate statistically significant differences ($P < 0.05$) of a sample compared to control (single asterisks) or to both the control and the lower MPTP dose (double asterisks).

Fornai et al. Parkinson-like syndrome induced by continuous MPTP infusion: Convergent roles of the ubiquitin-proteasome system and α-synuclein. Proc Natl Acad Sci U S A. 2005 March 1;102(9):3413-3418.



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Effect of an α-synuclein deletion on MPTP toxicity. (b) Uptake of [14C]2-DG in littermate wild-type and α-synuclein KO mice that were continuously infused for 7 days with control or MPTP (30 mg/kg daily) solution. Pictures display false-color autoradiograms. (c) Proteasome activity in control and α-synuclein KO mice continuously infused with MPTP (30 mg per kg of body weight daily). Proteasome activities in the substantia nigra are depicted as percent of control (means ± SEMs) as a function of time after beginning of the infusions (five mice per group). In a and c, asterisks indicate statistically significantly different values ($P < 0.05$) from controls.

Fornai et al. Parkinson-like syndrome induced by continuous MPTP infusion: Convergent roles of the ubiquitin-proteasome system and α-synuclein. Proc Natl Acad Sci U S A. 2005 March 1;102(9):3413-3418.

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N/A, Neuroinflammation leads to Degeneration of dopaminergic neurons of the nigrostriatal pathway (https://aopwiki.org/relationships/906)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Rodent models have been mainly used to study the impact of neuroinflammation on DAergic nigrostriatal pathway degeneration, without any sex restriction. Neuroinflammation preceding neuronal death was detected in monkeys exposed to MPTP (Barcia et al., 2011); and in human, neuroinflammation is considered as an early event in the disease process (Innaccone et al., 2012).

How Does This Key Event Relationship Work

Cells of the innate (microglia and astrocytes) and adaptive (infiltrating monocytes and lymphocytes) immune system of the brain have, like other immune cells (in peripheral tissues), various ways to kill neighboring cells. This is in part due to evolutionary-conserved mechanisms evolved to kill virus-infected cells or tumor cells; in part it is a bystander phenomenon due to the release of mediators that should activate other cells and contribute to the killing of invading microorganisms. An exaggerated or unbalanced activation of immune cells can thus lead to parenchymal (neuronal) cell death (Gehrmann et al., 1995). Mediators known to have such effects, and that are also known to be produced during inflammation in the brain comprise components of the complement system and cytokines/death receptor ligands triggering programmed cell death (Dong and Benveniste, 2001). Besides these specific signals, various secreted proteases (e.g. matrix metalloproteases), lipid mediators (e.g. ceramide or gangliosides) or reactive oxygen species can contribute to bystander death of neurons (Chao et al., 1995; Nakajima et al., 2002; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and Block, 2013). Especially the equimolar production of superoxide and NO from glial cells can lead to high steady state levels of peroxynitrite, which is a very potent cytotoxicant (Yuste et al., 2015). Already damaged neurons, with an impaired anti-oxidant defence system, are more sensitive to such mediators. An important role of microglia in the brain is the removal of cell debris (Xu et al., 2015). Healthy cells continuously display anti-“eat me” signals, while damaged and stressed neurons/neurites display “eat-me” signals that may be recognbized by microglia as signal to start phagocytosis (Neher et al., 2012), thus accelerating the loss of DA neurites in the striatum. Activated microglia surrounding DAergic neurons in PD express the M1 neurodegenerative phenotype (Hunot et al., 1999), which promote proliferation and function of CD4+ T cells (for review Appel et al., 2010), which in turn induce DA neuron toxicity, as assessed by experiments with immunodeficient mice (Brochard et al., 2009). Possible infiltration of other myeloid cells, such as monocytes or macrophages through a compromised blood-brain barrier, may also be involved in phagocytosis and neurodegeneration (Depboylu et al., 2012 ; Pey et al., 2014).

Weight of Evidence

Biological Plausibility
Histopathological studies have shown that glial activation is a hallmark of every neurodegenerative disease, including Parkinson’s disease (Whitton, 2007 ; Tansey and Goldberg, 2009 ; Niranjan, 2014 ; Verkhratiky et al., 2014). PET studies in PD patients have revealed that microglial activation in the substantia nigra is an early event in the disease process (Iannaccone et al., 2012), and that it is extremely persistent. The role of astrocytes is less clear than the one of microglia, but reactive astrocytes are able to release neurotoxic molecules (Mena and Garcia de Ybenes, 2008 ; Niranjan, 2014). However, astrocytes may also be protective due to their capacity to quench free radicals and secrete neurotrophic factors. The activation of astrocytes reduces neurotrophic support to neurons, and the proportion of astrocytes surrounding dopaminergic neurons in the substantia nigra is the lowest for any brain area suggesting that dopaminergic neurons are more vulnerable in terms of glial support (for review, Mena and Garcia de Ybenes, 2008). In vitro co-culture experiments have demonstrated that reactive glial cells (microglia and astrocytes) can kill neurons (Chao et al., 1995 ; Brown and Bal-Price, 2003 ; Kraft and Harry, 2011 ; Taetzsch and Block, 2013), and that interventions with e.g. i-NOS inhibition can rescue the neurons (Yadav et al., 2012; Brzozowski et al., 2015). Direct activation of glial cells with the inflammogen LPS has also resulted in vivo in the death of DA neurons (Sharma and Nehru, 2015; Zhou et al., 2012; Li et al., 2009).

Circulating monocytes and lymphocytes: Neuroinflammation can disrupt blood-brain barrier integrity (Zhao et al., 2007), facilitating infiltration of circulating monocytes and lymphocytes (Machado et al., 2011; Quian et al., 2010). T cell infiltration has been found in CNS tissue of PD patients (Miklossy et al., 2006 ; Qian et al., 2010), and in animal models, in which depletion or inactivation of lymphocytes has been found to protect striatal DA terminals (for review, Appel et al., 2010).

Empirical Support for Linkage
LPS injections: Lipopolysaccharide (LPS, a known activator of microglia) injected into the substantia nigra successfully replicated the pathogenic features of Parkinson’s disease in rats. An increase in the mRNA expression of pro-inflammatory cytokines (TNF-alpha, IL-1 beta) was observed 7 days post-injection; alterations

in oxidative stress markers (ROS, lipid peroxidation, NO formation, NADPH oxidase activity, GSH system, SOD and catalase) became significant 14 days post-injection, and this was followed by a significant decline in tyrosine hydroxylase (TH), as marker of dopaminergic neurons (Sharma and Nehru, 2015). LPS-induced downregulation of TH expression seemed to depend on the pro-inflammatory cytokine IL-1 beta, since it was not observed in LPS-injected IL-1 knockout mice (Tanaka et al., 2013). Progressive hypokinesia, selective loss of dopaminergic neurons in substantia nigra and reduction of striatal dopamine content, as well as alpha-synuclein aggregation in substantia nigra was also achieved by unilateral intranasal instilation of LPS every other day for 5 months, mimicking a progressive inflammation-mediated chronic pathogenesis of Parkinson's disease (He et al., 2013). It is important to note that LPS administrated either directly in the brain, intraperitoneally or in utero results in a delayed and progressive loss of nigral DA neurons that persists well after the initial inflammatory stimulis (for review, Taetzsch and Block, 2013).

Rotenone: Chronic systemic rotenone exposure reproduces features of Parkinsons' disease with loss of DA neurons and putative Lewis bodies in substantia nigra, accompanied by neuroinflammation and oxidative stress, and reduction of TH immunoreactivity in striatum together with an increase in reactive astrocytes (Betarbet et al., 2000; Ferris et al., 2013). In this chronic rotenone model (2-3 mg/kg per day up to 4 weeks), microglia activation precedes neuronal death (Sherer et al., 2003). Several interventions aiming at blocking several features of microglial activation (NADPH oxidase, myeloperoxidase, phagocytosis, opening of K ATP channels, …) protected DA neurons from death (Wang et al., 2014 ; Emmrich et al., 2013 ; Chang et al., 2013 ; Salama et al., 2013 ; Zhou et al., 2007 ; Gao et al., 2003). An enhanced sensitivity of dopaminergic neurons to rotenone-induced toxicity was observed with aging, in parallel with the increase of glial cell activation in older rats (Phiney et al., 2006). In vitro, little neurotoxicity was detected in primary DA neuron cultures (low glia-content) exposed to rotenone, whereas significant and selective dopaminergic neurodegeneration was observed in neuron/glia cultures (Gao et al., 2002).

MPTP/MPP+: Following MPTP treatment, microglial cells are activated by a mechanism secondary to dopaminergic neuron injury (Zhou et al., 2005). However, elevation of interferon-gamma and TNFalpha in substantia nigra was detected before the death of DAergic neurons (Barcia et al., 2011); and serum levels of IFN-gamma and TNFalpha remain elevated for years in monkeys exposed to MPTP (Barcia et al., 2011). The role of microglia in the progression of DA neurodegeneration is suggested by in vivo and in vitro experiments in which feature of microglial reactivity (TNF-alpha, i-NOS, NADPH-oxydase, ROS generation) were blocked (Brzozowski et al., 2015; Wang et al., 2006 ; Liu et al., 2015 ; Wang et al., 2014 ; Chung et al., 2011 ; Borrajo et al., 2014 ; Bodea et al., 2014 ; Sriram et al., 2002 ; Feng et al., 2002 ; Dehmer et al., 2000 ; Ferger et al., 2004). Some evidence from above studies also extends to astrocytes (Sathe et al., 2012; Khan et al., 2014). For instance, systemic adminstration of nicotine (stimulating the anti-inflammatory role of alpha 7 nicotinic acetylcholine receptors on astrocytes and microglia) reduced MPTP-induced motor symptoms, and protected against neurodegeneration in the substantia nigra by (Liu et al., 2012; 2015). Entrance into the brain of bone marrow-derived cells expressing i-NOS may also play a deleterious role in neurodegeneration (Kokovay and Cunningham, 2005). Indeed, pharmacological inhibition or deletion of CD95 in peripheral myeloid cells hampered brain infiltration and was protective for MPTP-induced DA loss in striatum (Gao et al., 2015 ; Chung et al., 2015). Similarly, therapies aiming at suppressing immune reactivity, such as administration of Treg cells (CD4+CD25+ regulatory T cells) lead in MPTP treated mice, to a robust nigrostriatal protection associated to an inhibition of microglial reactivity (Reynolds et al., 2010).

A deleterious role of type-1 interferons (key modulators of early neuroinflammation), was demonstrated in mice treated with MPTP. Mice lacking the type-1 IFN receptor showed an attenuated pro-inflammatory response and reduced loss of dopaminergic neurons and the neuroprotective potential was confirmed by treatment with a blocking monoclonal IFNAR1 antibody (Main et al. 2016).

Paraquat: Paraquat alone (10mg/kg, 2x/week, for 4 weeks) or in combination with maneb (30 mg/kg) induces a loss of DAergic neurons in the substantia nigra paralleled by an increase in microglial reactivity (Cicchetti et al., 2005; Mitra et al., 2011). In a paraquat rat model, microglial reactivity was observed 4 weeks post-injection, whereas degeneration of DAergic neurons was only observed 2 weeks later (Sant-Pierre et al., 2006). Direct treatment of primary microglial cells with paraquat (5-15 microM) showed no morphological change and no upregulation of IL-10, IL-1beta, IL-2 , IL-4, TNF-alpha, GM-CSF or INF-gamma, suggesting that paraquat cannot activate directly microglial cells (Klintworth et al., 2009), despite contrasting observations in the microglial cell lines BV2 (Miller et al., 2007) or N9 (Bonneh-Barkay et al., 2005). But « priming » of microglial cells by a first exposure to paraquat (10 mg/kg) (Purisai et al., 2007), by LPS (2-4 mg/kg) (Purisai et al., 2007), or by a viral mimic (Bobyn et al., 2012) increased the vulnerability of DA neurons to further paraquat treatments. Interestingly, if minocycline (45 mg/kg), an antibiotic known to decrease microglial reactivity, was applied together and after the first priming paraquat treatment, subsequent exposure to paraquat failed to cause DA neurodegeneration (Purisai et al., 2007). If paraquat treatments were made in mice lacking functional NADPH oxidase, no DA neurodegeneration was detected (Purisai et al., 2007), identifying again NADPH-oxidase as a key factor (Wu et al., 2005). In particular, the NADPH oxidase isoform NOX2 located on microglia plasma membranes transfers electrons to paraquat inducing the formation of the paraquat radical cation (Rappold et al. 2011). Radical paraquat may then (i) react with oxygen efficiently producing superoxide and regenerating paraquat, and/or (ii) enter DA neurons being a substrate for the dopamine transporter (DAT) (Rappold et al., 2011). This second possibility is supported by the observation that cells expressing DAT efficiently uptake paraquat only in the presence of microglia, but not when NOX2 activity is specifically abolished (Rappold et al. 2011). Neurodegeneration may be then triggered (i) by the amplification of the extracellular redox signalling

(Purisai et al., 2007, Bonneh-Barkay et al., 2005) and/or (ii) establishing a new round of redox cycling once paraquat is taken up into DA neurons. Accordingly, expression of DAT sensitize HEK293 cells to paraquat (50 microM) induced intracellular ROS production and cell death as well as mutant mice with hypomorphic DAT are resistant to paraquat neurotoxicity (Rappold et al. 2011). Besides NADPH-oxidase, other inflammatory factors are involved in DA neurodegeneration : for example, iNOS, NF-kappaB or p38 MAPK, since their blockade reverted the 50% decrease of TH immunoreactivity, as well as IL1-beta and NO increased expression in striatum observed following paraquat or paraquat and maneb treatments (Yadav et al., 2012). Similarly, IFN-gamma silencing prevented the paraquat-induced morphological signs of microglial activation, the NADPH-oxidase expression, as well as the time-dependent changes in the pro-inflammatory enzymes i-NOS and COX-2, of cytokines (IL-1beta, TNF alpha), and of signaling molecules (JNK and p38 MAPK), and protected against paraquat-induced DA neurodegeneration (Mangano et al., 2012). Protection against paraquat-induced DA neurodegeneration can also be achieved by providing trophic support (intranigral or peripheral injection of GDNF or GM-CSF, respectively), which is reduced upon paraquat treatment (Mangano et al., 2011).

Uncertainties or Inconsistencies

• Mice deficient in microglia (depletion by a ganciclovir-thymidine kinase system under the CD11b promoter) were still susceptible to MPTP toxicity, while mixed cell cultures prepared from these deficient mice showed partial protection (Kinugawa et al., 2013).

• Although some publications show strong protection by COX-2 inhibition/deletion, others showed that mice deficient for COX-2 were partly protected against MPTP-induced decrease of DAergic neurons in substantia nigra, but not against DA terminal loss in striatum (Feng et al., 2000).

• Mice deficient in IL6 (IL6-/-) showed an increased vulnerability of the nigrostriatal pathway following MPTP treatment associated to a normal astrogliosis but a transient microgliosis, suggesting that transient microgliosis and IL6 may have also protective effects (Cardenas and Bolin, 2003).

• MMTV integration site 1 (Wnt 1) is a key transcript involved in DAergic neurodevelopment, and is dynamically regulated during MPTP-induced DA degeneration and glial activation. MPTP-activated astrocytes of the ventral midbrain were identified as candidate source of Wnt 1 by in situ hybridization and RT-PCR in vitro, suggesting that reactive astrocytes may be rather involved in neuroprotective/neurorescue pathways, as further demonstrated by deletion of Wnt 1 or pharmacological activation of Wnt/ -catenin signaling pathway (L'Episcopo et al, 2011).

• The role of microglia, NADPH-oxidase and oxidative stress in paraquat-induced neurodegeneration is well established. Nevertheless, the mechanism connecting these three elements remain poorly understood since direct evidence for extracellular and/or intracellular formation of radical paraquat and superoxide is controversial.

• Rotenone (1-3 nM) applied directly on BV2 microglial cells increased their phagocytosis and the release of pro-inflammatory cytokines (TNF-alpha, IL-1 beta), suggesting that microglial cell can also be a primary target of rotenone (Zhang et al., 2014). However, these results in a transformed microglial cell line contrast with the experiments performed on isolated primary microglial cells, where rotenone (10-50 nM) was not able to trigger a direct activation (Klintworth et al., 2009).

• The regulation of inducible nitric oxide synthase (for production of peroxynitrite) differs strongly between rodents and human, and thus, the role of NO in human remains unclear (Ganster et al., 2001).

• While in human long-term use of anti-inflammatory drugs (NSAIDs, aspirin, iboprufen) for preventing PD onset or for slowing the progression is still controversial, a new strategy is emerging aiming at targeting microglial cells by modulating their activity, rather than simply trying to counteract their inflammatory neurotoxicity. The advantage of this therapeutic approach could be to reduce neuroinflammation and neurotoxicity, while at the same time strengthening intrinsic neuroprotective properties (Pena-Altamira et al., 2015)

Quantitative Understanding of the Linkage

As it is rather the features and the duration of the inflammatory response that determine the extent of the nigrostriatal pathway neurodegeneration, the best way to propose a quantitative or semi-quantitative evaluation of the links between KEup and KEdown is to use studies where any feature of neuroinflammation is inhibited and to quantify the protection of the Daergic neurons and terminals. Thus it will give an evaluation of how much neurodegeneration depends on the neuroinflammatory process. Below are some examples for illustration.

KE upstream **Neuroinflammation**	**KE downstream** **Neurodegeneration of dopaminergic nigrostriatal pathway**	**Reference**	**Type of study**	**Comment**
Inhibition of any feature of neuroinflammation (microglia/astrocyte)	***How much nigrostriatal pathway degeneration depends on KEup*** ***as assessed by protection when any KEup feature is inhibited***			

KATP channel opener (iptakalim) induced decrease of TNF-alpha and COX2 mRNA expression and TNF-alpha content, as well as microglial reactivity (OX42, ED1)	TH immunoreactivity : Total recovery	Zhou et al., 2007	In vivo Rotenone 2.5 mg/kg/d + in vitro	
NADPH oxydase Neuron enriched cultures Neuron-Glia co-cultures +apocynin	DA uptake TH immunoreactivity About 50% more neuronal death in presence of glia (80 % of protection with apocynin)	Gao et al., 2002	In vitro Rotenone 5-20 nM	
NADPH oxydase Mice knockout for NADPH ox gp91-/- Co-culture neuron-glia	DA uptake : 40% protection TH immuno : 20% protection	Gao et al., 2003	In vitro Rotenone 5-10 nM	
Phagocytic signaling between neuron and microglia i.e. block of vitronectin and P2Y6 on microglia or annexin or phophatidylserine on neuron (eat-me signal)	About 20% neuronal protection	Emmrich et al., 2013	In vitro Co-cultures of cerebellum Rotenone 2.5 nM	
Decrease in the number of activated microglia by **L-thyroxin** in substantia nigra, not in striatum	Protection of DA terminals in striatum, but no effect in substantia nigra	Salama et al., 2012	In vivo Rotenone 3mg/kg/d	
Myeloperoxidase (HOCl from H2O2) Resveratrol decreased NO, ROS, phagocytosis in microglia and astrocytes	Protection of neuron : 40% cell viability 50-60% TH immuno + number of dendrites	Chang et al., 2013	In vitro Rotenone 30 nM MPP+ 0.1 microM	
NADPH oxydase : NOX2 Diphenyleneiodonium : long acting NOX2 inhibitor	DA uptake and TH immuno : 30-40 % of protection	Wang et al., 2014	In vitro LPS 20 ng/ml MPP+ 0.15 microM	
Control of microglial and astrocyte reactivity by Alpha 7 nicotinic Ach receptor present on microglia and astrocyte Its activation decreased microglial and astrocyte reactivity	MPP+ cuased 40% decrease of TH+ neurons Nicotine induced a 30% recovery	Liu et al., 2012, 2015	In vivo MPTP 20mg/kg Nicotine 5mg/kg In vitro on isolated microglia and astrocytes	
""***TNF-alpha of microglial origin*** By blocking angiotensin-1 receptors, NADPH-oxydase, Rho-kinase and NF.kB	20 % of recovery of TH immunoreactivity	Borrajo et al., 2013	In vitro + in vivo MPP+ 0.25 microM	
Infusion of the anti-inflammatory cytokine TGF beta protects from MPP+-induced cell loss by decreasing CD11b, i-NOS, TNFalpha, IL+ beta, and increas ing IGF-1. Silencing of TGFbR1 gene abolished the protective effect	MPP+ caused 60% decrease of TH immuno, and TGFbeta induced a dose-dependent recovery (5-20 ng/ml)	Liu et al., 2015	In vitro Co-cultures MPP+ 5 microM	indirect

i-NOS inhibition caused a decrease of astrocyte and microglial reactivity as assessed by GFAP and OX6, respectively (n-NOS inhibition had no effect)	TH immunoreactivity Dose-dependent recovery with 1400W (0.1-100 micoM)	Brzozowski et al., 2015	In vitro MPP+ 43 microM	
Inhibition of laminin receptor on microglia i.e. regulating cell-ECM interactions induced a decrease of microglia phagocytosis and of O2- production	Dose-dependent partial recovery (about 35% of TH immunoreactivity	Wang et al., 2006	In vitro MPP+ 0.1-0.5 microM	
Inhibition of glial activation-mediated oxidative stress by Fluoxetine, anti-depressant)	30% of recovery of TH immunoreactivity in Substantia nigra and total recovery of DA terminals in striatum	Chung et al., 2011	In vivo MPTP 20 mg/kg ip	
Mice lacking both TNFR Induced a decrease of GFAP in striatum Double KO, if only KO for TNFR1 or TNFR2, no protection	TH staining in striatum, DA content and GFAP staining , all returned to control level	Sriram et al., 2014	In vivo MPTP 12.5 mg/kg sc	
Mice-deficient for COX2 Microglial cells are the major cells expressing COX2	MPTP caused in substantia nigra 40% loss in wild type 45% loss in COX1-/- 20% loss in COX2-/- in striatum 70% loss of DA in all 3 types of mice	Feng et al., 2002	In vivo MPTP 20 mg/kg sc	
S100B-/- in astrocytes caused decreased microgliosis, TNF-alpha and RAGE	12% of protection for TH+ neuron 30% of protection for Nissl-labelled neurons	Sathe et al., 2012	In vivo MPTP 30 mg/kg ip	
Glia Maturation Factor (GMF) overexpression or **GMF-/-** showed decreased TNF-alpha, IL-1beta, ROS and NFkappaB downregulation	Overexpression of GMF exacerbate DA neuron degeneration GMF-/- induced a protection of 40% of TH+ neurons	Khan et al., 2014	In vitro Mesencephalic neuron/glia cultures MPP+ 5,10,20 microM	
Pharmacological inhibition or deletion of CD95 in peripheral myeloid cells (monocytes, macrophages, microglia, leucocytes) hampered infiltration in the brain of peripheral myeloid cells	Total preservation of DA level in striatum Total protection of TH+ neurons in Snigra (25% affected in wild type mice)	Gao et al., 2015	In vivo MPTP 30 mg/kg ip	
'Glucocorticoid receptor (GR) deletion in microglia increased their reactivity and induced a persistant activation	2X aggravation of TH+ neuronal loss in Snigra	Ros-Bernal et al., 2011	In vivo MPTP 20 mg/kg ip	
TNF -/- mice	No protection in substantia nigra TH density in striatum : return to control level	Ferger et al., 2004	In vivo MPTP 20 mg/kg ip	

Intra-venous transplantation of mesenchymal stem cells Cell migration in substantianigra and release of TGFbeta (anti-inflammatory) Reparation of BBB, decreased infilatration and microglial activation	About 15% protection of TH+ neurons in Snigra	Chao et al., 2009	In vivo MPTP 20 mg/kg ip	
Nrf2-/- Increase in microgliosis and astrogliosis Microglial M1 phenotype Nrf2 involved in tuning microglial activation, switch M1/M2 phenotypes	40% more DA neurons loss in substantia nigra (TM immunostaining)	Rojo et al., 2010	In vivo MPTP 20mg/kg ip	indirect
Beta2 adrenergic receptor activation decreased microglial activation	20% protection of TH+ neurons in Substantia nigra	Qian et al., 2011	In vivo MPTP 15 mg/kg sc	
Deficiency in i-NOS blocks MPTP-induced increase of i-NOS, but not morphological microglial activation (IB4)	Rescue of TH+ neurons in substantia nigra to control level, but no protection for striatal DA content	Dehmer et al., 2000	In vivo MPTP 30 mg/KG/d ip, 5d	
C3-deficient mice Inhibition of complement-phagossome pathway Induced a decrease in several markers of microglial activation	Loss of DA neurons induced by repeated systemic LPS application is rescued to control level	Bodea et al., 2014	In vivo 4 dayly injection of LPS 1 microg/gbw LPS	
Minocyline or silencing of NADPH oxidase Microglial priming by a sigle injection of paraquat (PQ) (10mg/kg) or by LPS (2-4 mg/kg) increased the vulnerability of DA neurons.	Blockade of priming by minocycline or by silencing NADPH oxidase prevent DA neurodegeneration by subsequent exposure to PQ.	Purisai et al., 2007	In vivo Paraquat 10 mg/kg	
Interferon-gamma knockout prevented PQ-induced microglial activation as evidenced by morphological changes, i-NOS, COX2, IL1beta, TNFalpha,overexpression	In the knockout mice, DAergic neurons were protected from PQ-induced neurodegeneration	Mangano et al., 2012	In vivo Paraquat 10 mg/kg	
Absence of microglia or NADPH silencing No effect of PQ on DA uptake and TH immunoreactivity in cultures depleted of microglia. No effect of PQ in neuron-glia co-cultures prepared from NADPH oxidase- deficient mice	Microglial NADPH oxidase as essential factor for mediating DA neurodegeneration	Wu et al., 2005	In vitro Paraquat 0.5 – 1 microM	
Blockade of i-NOS, NF-kB or p38 MAPK Cause a significant decrease of microglial reactivity , NO and IL-1beta	TH immunoreactivity, recovery of 20%	Yadav et al., 2012	In vivo Paraquat 10 mg/kg ip ± maneb 30 mg/kg	

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Degeneration of dopaminergic neurons of the nigrostriatal pathway leads to N/A, Neuroinflammation (https://aopwiki.org/relationships/907)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Beside the rodent models, the concept of vicious circle with neuronal injury leading to neuroinflammation and neuroinflammation triggering or enhancing neurodegeneration is described in several neurodegenerative diseases in human, without any sex restriction (Hirsch and Hunot, 2009; Tansey and Goldberg, 2009; Griffin et al., 1998; McGeer and Mc Geer, 1998; Blasko et al., 2004; Cacquevel et al., 2004; Rubio-Perez and Morillas-Ruiz, 2012; Thundyil and Lim, 2014; Barbeito et al., 2010). Aging is an aggravating factor and increases the risk for developing a neurodegenerative disease (Kawas et al., 2000; Blasko et al., 2004).

How Does This Key Event Relationship Work

Several chemokines and chemokines receptors (fraktalkine, CD200) control the neuron-microglia interactions and a loss of this control on the side of neurons can trigger microglial reactivity without any further positive signal required (Blank and Prinz, 2013; Chapman et al., 2000; Streit et al., 2001). Upon neuronal injury, signals termed “Damage-Associated Molecular Patterns (DAMPs)” are released by damaged neurons to promote microglial reactivity (Marin-Teva et al., 2011; Katsumoto et al., 2014). These are for instance detected by Toll-

like receptors (TLRs) (for review, see Hayward and Lee, 2014). TLR-2 functions as a master sensing receptor to detect neuronal death and tissue damage in many different neurological conditions including nerve transection injury, traumatic brain injury and hippocampal excitotoxicity (Hayward and Lee, 2014). Astrocytes, the other cellular actor of neuroinflammation besides microglia (Ranshoff and Brown, 2012) are also able to sense tissue injury via e.g. TLR-3 (Farina et al., 2007; Rossi, 2015), and neuronal injury can result in astrocytic activation (Efremova, 2015).

The SNpc can be particularly vulnarable to the inflammatory process; its contains more microglia than astrocytes when compared with other areas of the brain and this can promote stronger neuroinfammation (Mena et al. 2008, Kim et al. 2000).

Weight of Evidence

Biological Plausibility

Kreutzberg and coworkers (1995, 1996) showed that neuronal injury generally leads to activation of microglia and astrocytes. This is a general phenomenon: for instance it is always observed in ischemic damage (stroke; often in the form of glial activation following neuronal injury (Villa 2007)) as well as in stab or freeze injuries (Allahyari and Garcia, 2015). It is also observed regularly when neurons are killed by highly specific neurotoxicants that do not affect glia directly, such as injection of quinolinic acid or of 6-hydroxydopamine into the striatum (Hernandez-Baltazar et al., 2013; Arlicot et al., 2014). The vicious circle of neuronal injury triggering glial activation and glial activation triggering/enhancing neurodegeneration is often assumed to be a key element in the pathogenesis of neurodegenerative diseases, not just PD, but also (Alzheimer's disease, prion disease and many others) (Hirsch and Hunot, 2009; Tansey and Goldberg, 2009; Griffin et al., 1998; McGeer and Mc Geer, 1998; Blasko et al., 2004; Cacquevel et al., 2004; Rubio-Perez and Morillas-Ruiz, 2012; Thundyil and Lim, 2014; Barbeito et al., 2010).

Innate immune system, mainly microglia and astrocytes is primary involved in Parkinson's disease the(Lucin et al. 2009, Glass et al. 20101, Rocha et al. 2012), and neurons are knowns to actively regulate the microglia response to stress (Mott et al. 2004, Cardona et al. 2006). Presence of reactive microglia has been observed in post-mortem brain tissue from PD patients or in people following intoxication with MPTP as well as in animal models of PD (McGeer et al. 1988, Langston et al. 1999, McGeer et al. 2003, Czlonkowska et al. 1996, Walsh et al. 2011). In co-cultures of neurons and microglia neuronal damage/cell death triggers microglia activation that potentiates MPTP-induced neuronal injury (Gao et al. 2003).

Empirical Support for Linkage

MPP+: The chemokine fractalkine (regulating neuron-glia interactions) was found to be released by neurons after unilateral injection of MPP+ in substantia nigra. It induced microglial activation by binding on the microglial receptor CXCR1 (Shan et al., 2011). Similarly, in chronically MPTP- injected macaques, metalloproteinases-9 (MMP-9) released by injured neurons favor glial activation (Annese et al., 2015). Advanced glycation endproducts (AGEs), which are endproducts of reactions involving ROS, colocalized with DAergic neurons 2 days post last MPTP injection, suggesting neuronal injury (Teismann et al., 2012). In contrast, the receptors for AGEs (RAGEs) were found on microglial cells and astrocytes (Teismann et al., 2012). RAGE can activate NF-kappaB, the transcription factor involved in the inflammatory response (Abdelsalam and Safar, 2015). Ablation of RAGE proved to be protective against MPTP-induced decreases of TH+ neurons, by decreasing NF-kappaB p65 nuclear translocation and by mitigating microglia and astrocyte reactivities (Teismann et al., 2012).

Rotenone: Rotenone-induced neurotoxicity was less pronounced in neuron-enriched cultures, than in neuron-glia co-cultures (Gao et al., 2002), sugggesting that neuron-glia interactions are critical for rotenone-induced neurodegeneration. Indeed, CD200-CD200R signaling regulates neuron-glia interactions and holds microglia in a quiescent state (Biber et al., 2007). Therefore, inhibition of CD200R by blocking antibodies increased rotenone-induced DA neurotoxicity in neuron-glia mesencephalic co-cultures (Wang et al., 2011). Aging is associated with a decrease of CD200 expression (Wang et al., 2011) and deficits in neuronal CD200 production is also observed in several animal models of Parkinson’s disease (Sung et al., 2012 ; Wang et al., 2011 ; Zhang et al., 2011). Inhibition of RAGE, which is upregulated in the striatum following rotenone exposure and in response to neuroinflammation, decreases rotenone-induced apoptosis by decreasing mitochondrial cytochrome c release and caspase-3 activation and suppresses NF-kappaB activation, as well as the downstream inflammatory markers TNF-alpha, i-NOS and myeloperoxidase (Abdelsalam and Safar, 2015), showing again intermingled links between neuronal injury/death and neuroinflammation.

Paraquat: Non-lethal neuronal damage is sufficient to trigger neuroinflammation: in 3D rat brain cell cultures, repeated treatment with concentrations of paraquat that did not kill the neurons, microglia and astrocytes were activated (Sandström et al., 2014). Paraquat alone (10mg/kg, 2x/week, for 4 weeks) or in combination with maneb (30 mg/kg) induces a loss of DAergic neurons in the substantia nigra paralleled by microglial activation (Cicchetti et al., 2005 ; Mitra et al., 2011). Neuronal injury is facilitated by uptake of paraquat via DA transporters (Rappold et al., 2011). In this model, paraquat-induced neuronal perturbations are sufficient to induce neuroinflammation, but then neuroinflammation exacerbates the neurodegenerative process (Purisai et al., 2007).

Uncertainties or Inconsistencies

• Triggering of glia by injured neurons may not necessarily be due to the damage of neurons, but it may also be due to released synuclein (Sanchez-Guajardo, 2010)

• In a AAV alpha-synucleinoptahy model, it was shown that cytoskeletal perturbation and accumulation of alpha-synuclein were sufficient to induce microglial reactivity, suggesting that neuroinflammation appears early in the disease process and is not a result triggered by cell death (Chung et al., 2009)

• Direct effects of toxicants on glia cannot be completely excluded. They have been reported for most toxicants in one or the other publication (rotenone, paraquat, MPP+) (Zhang et al., 2014; Rappold et al., 2011; Brooks et al., 1989). The overwhelming evidence speaks against such effects for rotenone and MPP+ (Klintworth et al., 2009), but for paraquat there is evidence of direct interaction with microglial membrane NADPH oxidase (Rappold et al., 2011).

• As paraquat has several MIE (Czerniczyniec et al., 2015; Rappold et al., 2011), these may involve both neurons and microglia.

Quantitative Understanding of the Linkage
Some examples of quantitative relationships between KEup and KEdown are given below. For KEdown Neuroinflammation, only the features measured are cited, as neuroinflammation is a complex KE involving several cell types and measured by changes in the expression /release of several markers

KE upstream ***Degeneration of DAergic nigrostriatal pathway***	**KE downstream** ***Neuro-inflammation***	**Compound**	**Reference**	**Comment**
about 25 % decrease of TH+ neurons 24h-72h post-injection	Microglial and astroglial reactivities in substantia nigra and striatum	**MPTP** 20mg/kg i.p. 4 injections at 2h intervals	Annese et al., 2013	MMP-9 released by neurons as trigger of neuroinflammation
about 60% decrease of TH+ neurons in subt nigra and of DA terminals in striatum 7days post-injection	increase in ED1+ cells (macrophagic microglia or invading monocytes)	**MPTP** 20 mg/kg i.p. 4 injections at 2h intervals	Chung et al., 2013	MMP-3-induced disruption of BBB
about 50% decrease of TH+ neurons	microglial and astroglial reactivity in substantia nigra and striatum	**MPTP** 30mg/kg i.p. each day during 5 days	Teisman et al., 2012	RAGE as trigger of neuroinfl.
about 50% decrease of DA content in striatum	increase of TNF-alpha (about 5X) and of i-NOS (about 8X) in striatum	**Rotenone** 1.5mg/kg s.c. for 21 days	Abdesalam and Safar, 2015	
about 40% decrease of TH+ neurons about 50% decrease of TH+ neurons	about 20% increase in microglial diameter as sign of activation microglial reactivity in substantia nigra	**Paraquat** 0.5-2 microM (neuron-glia co-cultures) 10 mg/kg i.p. twice a week for 4 weeks	Cicchetti et al., 2005	
decrease of TH immuno-reactivity of about 50% in subst. nigra 60% in frontal cortex 60% in hippo-campus	IL-1beta immunoreactivity increased in frontal cortex and hippocampus TNFalpha imunoreactivity increased in all 3 regions Iba+ immunoreactivity increased in substantia nigra and decreased in frontal cortex and hippocampus	**Paraquat** 10 mg/kg i.p., twice a week for 4 weeks	Mitra et al., 2011	

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N/A, Mitochondrial dysfunction 1 leads to Degeneration of dopaminergic neurons of the nigrostriatal pathway (https://aopwiki.org/relationships/908)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**indirectly leads to**	**Moderate**	**Weak**

Evidence Supporting Applicability of this Relationship

There are no sex or age restiction for the applicability of this KEr and mitochondrial are essential for most of eukariotyc cells. Rotenone and MPTp have been tested successfully in primates and mice. The mouse C57BL/6 strain is the most frequently used strain in the reported experiments. A difference in vulnerability was observed, particularly for rats, depending on the strain and route of administration. The Lewis strain gives more consistency in terms of sensitivity when compared to the Sprague Dawley. In addition to rodents, the pesticide rotenone has been also studied in Caenorhabditis elegans (C.elegans), Drosophila, zebrafish and Lymnaea Stagnalis (L.stagnalis) (Johnson et al., 2015), indicating that the system is preseved across species.

How Does This Key Event Relationship Work

Neurons are characterized by the presence of neurites, the formation of action potentials, and the release and re-uptake of neurotransmitters into the synaptic cleft. The presence of long extensions implies a significant enlargement of total cell surface. In combination with the transmission of action potentials that require a continuous maintenance of active transport processes across the membrane, the steady state energy demand of these neurons is significantly higher compared with non-neuronal cells. Dopaminergic (DA) neurons located in the substantia nigra pars compacta (SNpc) that project into the striatum are unique with respect of the total length of their neurites and the number of synapses that are significantly higher compared with other neuronal cell types (Bolam et al., 2012). Besides this complex morphology DA neurons have a distinctive physiological phenotype that could contribute to their vulnerability (Surmeier et al., 2010). Other features such as high energy demand, high calcium flux, dopamine autoxidation process as well as high content of iron and high content of microglia makes these DA neurons at vulnerable population of cells to oxidative stress produced by mitochondrial dysfunction. These architectural features of SNpc DA neurons render this cell type as particularly vulnerable to impairments in energy supply. Mitochondrial dysfunction, either evoked by environmental toxins such as the complex I inhibitor rotenone or MPTP, by oxidative modifications of components of the mitochondrial respiratory chain, or by genetic impairments of mitochondrial ATP generation hence have direct influence on the function and integrity of SNpc DA neurons.

Weight of Evidence

Biological Plausibility

Mitochondria are organelles essentials for multiple cellular processes, including production of ATP, maintenance of calcium homeostasis, management of ROS production and apoptosis. Mitochondrial dynamics are also critical for the maintenance of cellular homeostasis, which involve multiple factors controlling mitophagy (Youle et al. 2012). Deregulation of mitochondrial functions may impact any neuronal population; however, SNpc DA neurons are indeed the most vulnerable population in PD. Multiple factors are related to their vulnerability: These include autonomous activity, broad action potentials, low intrinsic calcium buffering capacity, poorly myelinated long highly branched axons and terminal fields, and use of a catecholamine neurotransmitter, often with the catecholamine-derived neuromelanin pigment (Sulzer et al. 2013; Surmeier et al.2010).

The above mentioned factors imply a significantly higher total cell surface and a high energy requirement in order to maintain the re-distribution of ions across the membrane following an action potential. In addition, SNpc DA neurons are characterized by significantly higher numbers of synapses compared with other neuronal types or with DA neurons of different anatomical localizations (Anden et al., 1966; Kawaguchi et al., 1990; Kita et al., 1994; Bevan et al., 1998; Wu et al., 2000; Tepper et al., 2004). In humans, ca. 10 times higher numbers of synapses compared with rats are expected, making human DA neurons particularly vulnerable (Bolam et al., 2012; Matsuda et al., 2009). These extreme bioenergetics demands pose SNpc DA neurons energetically “on the edge”. Any stressor that might perturb energy production would hence lead to conditions under which the energy demand would exceed energy supply, resulting in cell damage and ultimately to cell death.

The mechanistic link between mitochondrial dysfunction and loss of SNpc DA neurons also comes from evidence of mutated proteins related to mitochondrial function in familial PD, resulting in reduced calcium capacity, increased ROS production, increase in mitochondrial membrane permeabilization and increase in cell vulnerability (Koopman et al. 2012; Gandhi et al. 2009). In addition, excessive ROS production can damage mitochondrial DNA and activate the intrinsic pathway of apoptosis (Tait et al. 2010). Additional sources of oxidative stress come from the autoxidation of dopamine and the active generation of ROS by activated glia cells; furthermore, the mitochondrial respiratory chain itself represents a source of constant superoxide formation, even under normal conditions (Moosmann et al., 2002).

Imbalance of mitochondrial dynamics have been also reported in a wide range of experimental models of PD and inhibition of the mitochondrial fission proteins (i.e. Drp1) promote mitochondrial fusion and fission and enhanced the release of dopamine from the nigrostriatal terminals (Tieu et al. 2014).

Additional link between mitochondrial dysfunction and the degeneration of DA neurons of the nigrostriatal pathway comes from studies indicating a reduced activity of mitochondrial complex I in human idiopathic PD cases in the substantia nigra (Keeney et al., 2006; Parker et al., 1989, 2008; Swerdlow et al., 1996). The impairment in complex I activity was directly correlated with an elevated sensitivity of SNpc DA neurons and their demise. Transfer of mitochondria from human platelets collected from idiopathic PD subjects into fibroblasts or neuronal cells resulted in elevated levels of basal oxidative stress, a declined supply with ATP, and an elevated vulnerability towards exogenous stressors such as the complex I inhibitors rotenone or the redox cycler paraquat (Swerdlow et al., 1996; Gu et al., 1998). Systemic application of complex I inhibitors such as rotenone or MPTP lead to a preferential loss of nigrostriatal DA neurons, while other brain areas or peripheral cells are not affected to the same degree (Langston et al., 1983).

Empirical Support for Linkage

The experimental support linking mitochondrial dysfunction with the degeneration of DA neurons of the nigrostriatal system is based on the analysis of mitochondria from PD patients, from genetic mouse models, from in vitro knockdown and overexpression systems, and from in vitro and in vivo toxin models.

- **In vitro/rotenone**: Prevention of ROS formation protects from cell death. The concept of mitochondrial dysfunction as a consequence of defects in complex I has been fueled by observations of impaired complex I activity in the SNpc, muscle, and in platelets of PD patients. Human neuroblastoma SK-N-MC cells, exposed to rotenone, displayed a time- and concentration-dependent decline in viability. Transfection of rotenone-insensitive single subunit NADH dehydrogenase (NDI 1) allowed a replacement of endogenous complex I activity. NDI 1 transfected cells showed no oxidative damage, no declined mitochondrial activity, or cell death. A significant amount of endogenously formed ROS at complex I was identified in SK-N-MC cells and in a chronic midbrain slice culture exposed to rotenone. Antioxidants such as -tocopherol prevented cell death evoked by rotenone, but not the rotenone-induced drop in ATP (Sherer et al. 2003).

- **In vitro/rotenone/MPP+**: Antioxidants protect from rotenone/MPP+ cell death. Analysis of post mortem nigrostriatal material from PD patients regularly revealed the presence of elevated levels of oxidative modified proteins, lipids, and DNA. These observations indicate an elevated formation of ROS in the cells affected by the disease and triggerd the concept of antioxidants as a potential intervention strategy to slow down the progression of PD. In MES23.5 cells, a reduction in viability, DA content, NADH levels, as well as an increase in ROS formation and elevated nuclear condensation was observed upon treatment with MPP+. Rosmarinic acid is well known for its radical scavenging activities and displayed a complete protection from MPP+-mediated cell death and rescued NADH levels. In addition, it lead to a partial protection from the loss of DA and resulted in a rate of nuclear condensation that was about half of that observed with MPP+ alone (Du et al. 2010). The flavonoid rutin has been demonstrated to protect from oxidative stress in 6-OHDA induced motor deficits in rats as well as to inhibit the formation of nitric oxide and proinflammatory cytokines (Khan et al., 2012). In a model of

SH-SY5Y cells, exposure to rotenone lead to a reduction in viability by ca. 50% that was almost completely protected in the presence of rutin. Rotenone-dependent increase of ROS formation and an elevation of intracellular Ca2+ was significantly dampened by the presence of rutin, similar to its rescue from rotenone-dependent decrease in mitochondrial membrane potential (Park et al., 2014). Comparable observations were made with the quinone triterpene celastrol that protected SH-SY5Y cells exposed to rotenone almost completely from cell death, from a rotenone-dependent elevation in ROS levels, and from a rotenone-dependent loss of the mitochondrial membrane potential (Choi et al., 2014).

- **In vitro/different complex I inhibitors**: Inhibition of complex I triggers oxidant formation and cell death. The majority of experimental PD studies were either conducted using rotenone or MPP+. In order to demonstrate that the concept of complex I inhibition and its ROS-mediated triggering of mitochondrial dysfunction and cell demise can be regarded as a general principle, alternative complex I inhibitors were applied to substantiate previous observations made with rotenone. In human SK-N-MC neuroblastoma cells, rotenone as well as the pesticides fenazaquin, fenpyroximate, pyridaben, tebufenpyrad, pyridaben were tested. In all cases, a time- and concentration-dependent decline in intracellular ATP and cell viability was observed. Expression of the rotenone-insensitive NADH dehydrogenase from Saccharomyces cerevisiae (NDI 1) prevented from the toxicity of the different complex I inhibitors completely. Rotenone- and pyridaben-dependent cell death was prevented by ca. 75 % by the presence of the antioxidant α-tocopherol.(Sherer et al., 2007).

- **In vitro/rotenone**: Mitochondrial dysfunction-dependent cell death is prevented by antioxidants. In a human neuroblastoma SH-SY5Y model, exposed either to the complex I inhibitors MPP+ or rotenone, the imine antioxidants iminostilbene, phenothiazine, phenoxazine in the low nanomolar concentration range partially protected from MPP+ or rotenone toxicity. A reduction in the membrane potential evoked by MPP+ and rotenone was completely prevented by these antioxidants (Hajteva et al., 2009).

- **In vitro/rotenone**: Circumvention of dysfunctional mitochondria protects from cell death. Assuming a direct causal relationship between complex I inhibition, mitochondrial dysfunction, and the demise of DA neurons, the circumvention of endogenous complex I by expression of the NADH dehydrogenase of Saccharomyces cerevisiae (NDI 1) provided initial evidence for the essential role of complex I inhibition in this sequence of events. As an alternative electron carrier, capable of transferring electrons from NADH to cytochrome c, methylene blue was identified. In hippocampal HT-22 cells, a rotenone-mediated reduction in the oxygen consumption rate was completely reversed by the addition of methylene blue. A rotenone-mediated decline in cell viability by 70 % was almost completely prevented by 0.1 µg/ml methylene blue . In rats, rotenone-mediated decline in striatal DA was entirely prevented by methylene blue, the observed elevation of ROS formation evoked by rotenone was reduced to control levels, and rotarod performance impairments evoked by rotenone were completely avoided by administration of methylene blue. These observations illustrate a causal relationship between dysfunctional mitochondria, the degeneration of nigrostriatal DA neurons, and impaired motor performance (Wen et al. 2011).

- In vivo/rotenone: Circumvention of dysfunctional mitochondria prevents from nigrostriatal cell degeneration. Circumvention of a dysfunctional complex I by the rotenone-insensitive NADH dehydrogenase NDI 1 in vivo and its influence on nigrostriatal DA neuron integrity was demonstrated in a rat model with an unilateral injection of a recombinant adeno-associated virus, carrying the NDI 1 gene into close special vicinity to the SNpc. The animals were treated with rotenone after the unilateral expression of NDI 1. NDI 1 almost completely prevented from the rotenone-mediated loss of TH staining in the SNpc and the striatum. Striatal DA levels that were reduced by ca. 50 % by rotenone, in the presence of NDI 1, DA levels were also almost identical to the values of untreated controls. These observations highlight a causal relationship between the inhibition of complex I and the degeneration of nigrostriatal DA neurons (Marella et al. 2008).

- **In vitro/DA**: Exogenously added oxidants lead to mitochondrial dysfunction and cell death. Next to an elevated formation of reactive oxygen species evoked by endogenous defects in complex I or in response to pharmacological inhibitors of complex I, nigrostriatal DA neurons are characterized by the neurotransmitter dopamine and its tendency to undergo autoxidation when exposed to physiological pH and oxygen tension conditions. To assess the role of DA-mediated oxidative stress as a cause of mitochondrial dysfunction and its influence on cell viability, PC12 cells were exposed to DA. The observed increase in intracellular ROS was completely reversed by the presence of the antioxidant N-acetly-cysteine (NAC). The amount of oxidative modified protein increased by DA treatment, its rise was completely prevented by the presence of NAC, and partially prevented by the presence of exogenously added GSH. DA-dependent PC12 cell death, decline in the transmembrane potential and in intracellular ATP, and decline in complex II/III activities were observed and were all completely prevented by the presence of NAC. (Jana et al., 2011). - **In vitro/ GSH depletion**: Oxidative stress causes mitochondrial dysfunction and neurodegeneration. Several reports indicated a declined activity of complex I in the brain, but also in muscle and platelets of PD patients. In order to investigate the mutual interaction between pro-oxidative conditions and complex I activity, a PC12 subclone was generated, allowing the inducible downregulation of -glutamyl-cystein synthetase involved in the synthesis of glutathione (GSH). This system allows a controlled decrease of intracellular GSH by ca. 50 % and a decrease in mitochondrial GSH by ca. 40 %. Under these conditions, intracellular and intramitochondrial ROS increased by ca. one third, mitochondrial complex I activity and ATP levels were reduced by ca. two thirds. The observed inhibition of complex I was completely reversed by DTT. These observations indicate that an impairment of complex I activity as a key event in the initiation of mitochondrial dysfunction and ultimately cell death, can be evoked by elevated levels of oxidants, respectively by a declined cellular antioxidant capacity (Jha et al., 2000).

- **In vitro/ GSH depletion**: Oxidative stress causes mitochondrial dysfunction and neurodegeneration. PD is characterized by the depletion of glutathione (GSH) in the SNpc. Declined cellular levels of GSH were reported to be associated with morphological changes of mitochondria (Perry et al., 1982; Jain et al., 1991). To investigate the influence of declined GSH levels, N27 cells were exposed to buthionine-S-sulfoximine (BSO), an inhibitor of glutamate cysteine ligase and hence of de novo GSH synthesis. The BSO concentration chosen allowed a reduction in intracellular GSH levels by 50 % in the absence of cell death. Chronic GSH depletion resulted in the S-nitrosation of complex I and its inhibition. Both effects were completely reversed by the addition of DTT (Chinta et al., 2006). - **Isolated mitochondria**: Exogenous oxidants cause mitochondrial dysfunction. In order to further address the aspect on how DA autoxidation contributes to mitochondrial dysfunction and DA neurodegeneration, isolated rat brain mitochondria were exposed to DA, resulting in an inhibition of complex I by ca. 30 % and in an inhibition of complex IV by ca. 50 %. Both activities of complex I and complex IV were completely protected from DA-dependent inactivation by the presence of GSH. These observations point to a direct inhibitory action of endogenous DA and its autoxidation derivatives on the activity of the mitochondrial respiratory chain. (Khan et al., 2005) - **In vitro/cybrid cells**: Sensitization of neuronal cells for degeneration by transfer of dysfunctional mitochondria. In a subclone of human neuroblastoma cells (SH-SY5Y), devoid of mitochondrial DNA, mitochondria from platelets of PD patients were transplanted. Analysis after 5-6 weeks in culture after transplantation of mitochondria indicated a 20 % reduction in complex I activity, a 2-fold increase in the basal formation of reactive oxygen species, and a ca. 2-fold higher sensitivity towards the mitochondrial PD toxin MPP+ (Swerdlow et al., 1996).

- **In vitro/cybrid cells**: Sensitization of neuronal cells for degeneration by transfer of dysfunctional mitochondria. In a subclone of the human A549 cell line, devoid of mitochondrial DNA, mitochondria of platelets from PD patients were transplanted. Complex I activity in platelets of PD patients displayed a reduction of 25 % compared with age-matched controls. After transplantation into the A549 cells, complex I activity was reduced by 25% in its activity (Gu et al., 1998).

- **In vivo**: Induction of mitochondrial dysfunction by Drp1 deletion leads to neuronal cell loss. Maintenance of functional mitochondria in a cell is regulated by fission/fusion processes that allow the elimination of damaged mitochondria and the spreading of intact mitochondria. Deletion of the central fission protein dynamin related protein 1 (Drp1) leads to an elimination in DA neuron terminals in the caudate putamen and to a loss of DA neuron cell bodies in the midbrain. In Drp1 deficient mice, mitochondrial mass decreases, particularly in axons (Berthet et al., 2014) - In vivo: Induction of mitochondrial dysfunction by Tfam knockdown leads to neuronal cell loss. Mitochondrial transcription factor A (Tfam) is a key regulator of mitochondrial biogenesis. Conditional knockout mice with a selective disruption of the gene for mitochondrial Tfam in DA neurons indicated a reduction in mtDNA levels and deficiencies in the respiratory chain in midbrain DA neurons that progressed to DA cell death. The demise of DA neurons in the SNpc was associated with the onset of PD symptoms such as a reduction in locomotor activity of these mice by ca. 30 %. The decrease in locomotor activity was reversed by L-DOPA treatment (Ekstrand et al., 2007).

- In vivo: MPTP dependent mitochondrial dysfunction and cell death is protected by PGC-1 overexpression. Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) is a key regulator of mitochondrial biogenesis and metabolism. Transgenic mice overexpressing PGC-1 show protection against MPTP intoxication (50 %). The SNpc in these mice is characterized by elevated levels of SOD2, Trx2. Resveratrol is a known activator of SIRT1, leading to enhanced PGC-1 gene transcription. In MPTP mice, resveratrol protected TH-positve neurons by 80% from cell loss (Mudo et al., 2012).

- **In vivo**: Prevention of mitochondrial dysfunction protects from nigrostriatal cell loss. In order to demonstrate the causative connection between complex I-dependent mitochondrial dysfunction and the degeneration of DA neurons, a series of in vivo experiments were conducted that indicated partial restoration by antioxidants or by compounds supporting a dysfunctional mitochondrial ATP generation. In MPTP challenged mice that additionally received Q10 treatment, a 37 % higher striatal DA level compared with the MPTP group was detected. TH positive staining in the striatum dropped by ca. 65 % after MPTP. In the MPTP + Q10 group, the loss in striatal TH staining was reduced to ca. 40 % compared with the untreated controls. (Beal et al., 1998). In MPTP challenged marmosets, TH positive cell body numbers were reduced by ca. 60 %, co-administration with ebselen resulted in a reduction of TH staining of only ca. 25 % (Moussaoui et al., 2000). In MPTP challenged mice, a reduction of striatal DA by ca. 70 % was detected. Co-treatment with creatine resulted in a reduction of DA levels of only 42 %. In the same setup, TH positive neuron number in the SNpc was reduced by 70 % in response to MPTP, in the presence of creatine, a drop of only 4 % was observed (Matthews et al., 1999).

- **In vivo/rotenone**: Antioxidants prevent from rotenone-dependent nigrostriatal cell death. Rotenone administered subcutaneously for 5 weeks (2.5 mg/kg/d) caused a selective increase in oxidative damage in the striatum as compared to the hippocampus and cortex, accompanied by massive degeneration of dopaminergic neurons in the substantia nigra. Antioxidant polydatin (Piceid) treatment significantly prevented the rotenone-induced changes in the levels of glutathione, thioredoxin, ATP, malondialdehyde and the manganese superoxide dismutase (SOD) in the striatum, confirming that rotenone- induced mitochondrial dysfunction resulted in oxidative stress (Chen et al., 2015).

- **In vivo/rotenone**: Degeneration of DA neurons depends on oxidative stress evoked by mitochondrial dysfunction. Many studies have shown that mitochondrial aldehyde dehydrogenase 2 (ALDH2) functions as a cellular protector against oxidative stress by detoxification of cytotoxic aldehydes. Dopamine is metabolized by monoamine oxidase to yield 3,4-dihydroxyphenylacetaldehyde (DOPAL) then converts to a less toxic acid

product by ALDH. The highly toxic and reactive DOPAL has been hypothesized to contribute to the selective neurodegeneration of dopamine (DA) neurons. In this study, the neuroprotective mechanism of ALDH2 was observed as overexpression of wild-type ALDH2 gene, but not the enzymatically deficient mutant ALDH2*2 (E504K), reduced rotenone-induced DA neuronal cell death. Application of a potent activator of ALDH2, Alda-1, was effective in protecting against rotenone-induced (100 nM, 24 hr exposure) apoptotic cell death in both SH-SY5Y cells and primary cultured substantia nigra (SN) DA neurons. These results were confirmed by in vivo studies. Intraperitoneal administration of Alda-1 to C57BL/6 mice treated with rotenone (50 mg/kg/day, oral administration for 14days) or MPTP (40 mg/kg/day, i.p. for 14 days) significantly reduced death of SN tyrosine hydroxylase-positive dopaminergic neurons. The attenuation of rotenone-induced apoptosis by Alda-1 resulted from decreasing ROS accumulation, reversal of mitochondrial membrane potential depolarization, and inhibition of activation of proteins related to mitochondrial apoptotic pathway. The present study demonstrates that rotenone or MPP+ induces DA neurotoxicity through oxidative stress. Moreover, Alda-1 is effective in ameliorating mitochondrial dysfunction by inhibiting rotenone or MPP+ induced mitochondria-mediated oxidative stress that leads t0 apoptosis (Chiu et al., 2015).

Human studies: PD patients were found to show striatal oxidative stress directly relating to the progression of disease severity (Ikawa et al., 2011), suggesting that oxidative stress may cause synaptic dysfunction. Indeed, interruption of the activity-driven local ATP synthesis by synaptic mitochondria (an auto-regulated mechanism) can impair synaptic function (Rangaraju et al., 2014).

Uncertainties or Inconsistencies

- Several in vitro studies applying rotenone to evoke mitochondrial dysfunction came to the conclusion that rotenone-dependent ROS formation, and not the rotenone-evoked drop in ATP is the primary cause for cell degeneration. These observations are largely based on experimental systems employing the rotenone insensitive NADH dehydrogenase NDI 1. Expression of NDI 1 protected rotenone exposed cells from degeneration. The presence of NDI 1 however results in a substitution of ATP. Endogenously expressed complex I is still present in these models and it can be assumed that rotenone exposure would still lead to a complex I-dependent formation of ROS that precludes the modeling of a precise cause-consequence relationship between either ATP depletion or elevated ROS levels with the demise of DA neurons.

- Several studies indicate a dominant role of ROS in the degeneration of DA neurons, based on models in which rotenone/MPP+ mediated mitochondrial dysfunction and cell degeneration was protected by the presence of exogenously added antioxidants. Maintenance of the endogenous redox potential however is a highly ATP-dependent process. Clear-cut separations between the respective contribution of ROS or the role of an inhibited mitochondrial ATP synthesis on the degeneration of DA neurons is hence difficult to postulate.

- Studies with chronic partial GSH depletions indicated that an experimental reduction of GSH/GSSG by ca. 50 % has no influence on cell viability. Reports involving rotenone and MPP+ however regularly observe degeneration of DA neurons under conditions of GSH depletion around 50 %. These observations indicate a more prominent role of the intracellular drop of ATP evoked by the complex I inhibitors in the process of cell degeneration.

- Studies in which oxidative stress is generated e.g. by the application of DA or 6-OHDA not only observed a challenge of the cellular redox potential, but also reversible and irreversible inhibitory mechanisms of mitochondrial respiratory chain complexes (nitration, S-nitrosation) that are accompanied by an inhibition of the respiratory chain in the absence of pharmacological complex I inhibitors. These observations illustrate the close mutual interaction between oxidative stress and the inhibition of mitochondrial respiration and point to a profound role of direct mitochondrial inhibition also under oxidative stress conditions.

- Mitochondrial dysfunction is generally associated with conditions of oxidative stress. Dysfunctional mitochondria can act as potent source of superoxide. Oxidative stress associated with PD however not only originates from mitochondrial ROS, but also from DA autoxidation and the Fenton reaction, as well as from inflammatory activated adjacent glia. Interpretations on the role of oxidative stress in DA neurons and its role in DA neurodegeneration is hence hampered by the fact that the respective origin of the reactive oxygen species formed (mitochondria, DA autoxidation, inflammation of glia cells) is rather difficult to identify and often shows overlappings (Murphy et al., 2009; Starkov et al., 2008, Cebrian et al., 2015).

- In PD patients, a reduction in complex I activity in the SNpc, but also in peripheral tissue and cells such as platelets, was reported. Studies with isolated mitochondria indicated that for efficient inhibition of mitochondrial ATP formation, an inhibition of complex I by ca. 70 % is necessary (Davey et al., 1996). Reports on the reduction of complex I activity in PD patients however repeatedly indicated an inhibition of only 25-30 % (Schapira et al., 1989; Schapira et al., 1990; Janetzky et al., 1994).

- Data available on the respective inhibition of the components of the respiratory chain are highly dependent on the experimental setup used. Analysis of mitochondrial respiratory chain complex activities in mitochondrial homogenates provide results different from data obtained with intact, isolated mitochondria. These aspects need to be considered in the interpretation of such data (Mann et al., 1992; Parker et al., 2008; Mizuno et al., 1989; Schapira et al., 1990; Cardellach et al., 1993)

Quantitative Understanding of the Linkage

Quantitative understanding for this KE relationship mainly comes from in-vitro and engineered systems, using rotenone and MPTP as main chemical stressors. A clear response- response effect is evident as well as

temporality was mainly supported by evidence that modulation of the KE up was attenuating or preventing the KE down. Evidence of dose relationship was limited, as most of the time a single, generally high, concentration was used.

KE 2 upstream	KE 4 downstream	Comments	Reference
Rotenone experiments			
Mitochondrial membrane potential reduced by 50 % upon rotenone treatment. Back to 80 % compared to controls in the presence of the flavonoid rutin. Intracellular Ca2+ elevated by a factor of 3 by rotenone, reduction to an increase of 1.5 in the presence of rutin. ROS increased by a factor of 6.5; increase of ROS by a factor of 2 in the presence of rutin.	Rotenone (10 µM) resulted in a reduction of cell viability by 50 %. In the presence of rutin, cell viability was only reduced by 10 % upon rotenone treatment	SH-SY5Y cells exposed to rotenone (10 µM) for 24 h. When applied alone, rutin displayed no toxic effects, up to 100 µM. Rutin was added to the cells 30 min prior rotenone at concentrations from 0-10 µM	Park et al., 2014
Mitochondrial membrane potential reduced by ca. 66 % upon rotenone treatment; in the presence of celastrol, reduction by ca. 55 %. ROS formation increased by a factor of 2 in the presence of rotenone; ROS increase by a factor of 1.5 in the presence of celastrol.	Cell viability was reduced by 50 % by rotenone; In the presence of the triterpene celastrol, cell viability was only reduced by ca. 10 %	SH-SY5Y + rotenone (10 µM). Celastrol (2.5 nM) was applied 90 min prior to rotenone. Cells were incubated with the two compounds for a period of 24 h.	Choi et al., 2014
	TH staining in the SNpc in arbitrary units: Control (25) Rotenone (14) Rotenone + NDI 1(22) TH staining in the striatum Control (70) Rotenone (40) Rotenone + NDI 1 (65) DA levels in the striatum: Control (2.5) Rotenone (1.3) Rotenone + NDI 1 (2.2)	5 month old male Sprague-Dawley rats (ca. 500 g) received intracerebral injection of recombinant adeno-associated virus with the NADH dehydrogenase NDI 1 gene. 45 days after virus injection, rats were treated with rotenone-loaded microspheres (poly(DL-lactide-co-glycolide). 100 mg rotenone /kg body weight s.c. With this method, HPLC analysis of plasma rotenone revealed levels of 2 µM 14 days after microsphere treatment, and 1 µM 60 days after microsphere treatment. Behavioral experiments and brain sample collection was conducted 30 days after rotenone treatment.	Marella et al., 2008
MPP+ experiments			

Decline in mitochondrial transmembrane potential by MPP+; 50 % prevention from this decline by rosmarinic acid. NADH levels were reduced by ca. 50 % in the presence of MPP+; loss of NADH was completely prevented by the presence of rosmarinic acid. ROS levels increased by 50 % in the presence of MPP+. Rosmarinic acid lead to a reduced increase of ROS by only 20 % compared with the untreated control.	Cell viability reduced by MPP+ by 30 %, complete protection by the presence of the antioxidant rosmarinic acid. Striatal DA content reduced by 40 % by MPP+ treatment, partially protected by rosmarinic acid back to a value of 25 % reduction compared with the untreated control.	MES23.5 cells exposed to MPP+ (200 µM) for 24 h. Rosmarinic acid (1 nM) was applied 30 min prior to MPP+ treatment.	Du et al. 2010
Reduction in mitochondrial membrane potential by 60 % (MPP+), by 50 % (rotenone), complete recovery by the co-incubation with ISB, PHT, PHO	SH-SY5Y + MPP+: Cell viability reduced by 66 %; ISB, PHT, PHO partially protected from cell death with a reduction in cell viability by ca. 20 % SH-SY5Y + rotenone: reduction in cell viability by 60 % Partial protection by ISB, PHT, PHO to a reduction in cell viability by 25-50 %. SH-SY5Y + BSO: Reduction in cell viability by 80 % ISB, PHT, PHO partially protected with a residual decline in cell viability by ca. 20 %	SH-SY5Y + MPP+ (200 µM) or rotenone (150 nM) or BSO (150 µM) for 60 h and 72 h. Antioxidants tested: Iminostilbene (ISB) Phenothiazine (PHT) Phenoxazine (PHO) The antioxidants were applied 2 h prior to rotenone, MPP+, or BSO treatment	Hajieva et al., 2009
""***Circumvention of endogenous complex I***			
wt cells exposed to rotenone: increase in carbonyl content as marker of oxidative stress by 100 %; completely prevented in NDI 1 expressing cells. In midbrain slice cultures exposed to rotenone: increase in carbonyl content by 20 % Rats exposed to rotenone: increase in carbonyl content: 27 % in the striatum, increase by 41 % in the midbrain	SK-N-MC cells: rotenone evoked cell death protected by ca. 90 % in NDI 1 expressing cells. Rotenone induced cell death prevented by 80 % by alpha-tocopherol (62.5 µM and 125 µM).	SK-N-MC human neuroblastoma cells transfected with the rotenone insensitive NADH dehydrogenase NDI 1; Cells were treated with rotenone (100 nM) for 48 h or with BSO (10 µM) for 24 h. When both compound were used in a combined experiment, cells were first treated with BSO (10 µM) for 24 h, then rotenone (10 nM) was added for additional 36 h.	Sherer et al., 2003

Application of the complex I inhibitors: Rotenone Fenazaquin Fenpyroximate Pyridaben Tebufenpyrad Pyridaben	Time and concentration-dependent cell death with rotenone and a series of other complex I inhibitors. NDI 1 expressing cells were resistant towards the different complex I inhibitors.	SK-N-MC human neuroblastoma cells expressing the rotenone-insensitive NADH dehydrogenase NDI 1 from saccharomyces cerevisiae. All complex I inhibitors applied were added at the concentrations: 10 nM, 100 nM, 1 µM. Pyridaben was applied at 1 µM, 10 µM, 100 µM. Viability was assessed after 48 h, ATP was detected after 6 h. Carbonyl content was detected after 24 h.	Sherer et al., 2007
Oxygen consumption rate doubled by MB in the absence of complex I inhibitor. Oxygen consumption reduced by 50 % by rotenone; completely reversed to control levels by the presence of MB. Complex I-III activity reduced by 95 % by rotenone. Reversed to control levels by the presence of MB.	HT22 cell viability reduced by 70 % by rotenone. In the presence of MB, reduction by only 10 % of cell viability was observed. In rats treated with rotenone, rotarod retention time was reduced by 50 % by rotenone. Completely reversed to control levels by the co-administration of MB. In rats, rotenone evoked a reduction of striatal DA by 50 %; completely reversed to control levels by MB Complex I-III activity in the striatum of rats was reduced by 50 %, residual inhibition of 10 % observed in rats that were additionally treated with MB	The study included: • Isolated rat heart mitochondria exposed to rotenone (5 µM) (instant treatment) • Hippocampal HT-22 cells exposed to rotenone (2-8 µM) for 24 h. • Rats receiving rotenone (5 mg/kg/day via osmotic minipumps for 8 days Test of methylene blue (MB) (10 and 100 ng/ml in isolated mitochondria; 1 and 10 µg/ml in HT 22 cells) to circumvent the complex I/III blockade	Wen et al. 2011
Cybrid cells with PD mtDNA display a reduction in complex I activity by 20 %.	Cybrid cells: increase in basal formation of reactive oxygen species by 80%. 2-times higher sensitivity towards MPP+ as stressor	SH-SY5Y cells devoid of mtDNA; fused with platelets from PD patients for mitochondria transfer: cybrid cells. Treatment with MPP+ (40 or 80 µM) for 24 h or 48 h	Swedlow et al., 1996
Oxidative stress causes mitochondrial dysfunction			

Isolated mitochondria: Exposure to DA: loss of ca. 50 % membrane potential. Completely protected by GSH or N-acetyl-cystein (NAC) Decline of mitochondrial respiration capacity by 90 %. In the presence of NAC or GSH, only a reduction by 25-30 % was observed. PC12 cells exposed to DA, then isolation and analysis of mitochondria: inhibition of complex I activity by ca. 50 %, prevented by co-incubation with NAC. Inhibition of complex II and III; prevented by NAC. Intact PC12 exposed to DA: Mitochondrial transmembrane potential reduced by ca. 50 %; prevented by NAC Intracellular ATP reduced by ca. 50 %; Cell death increased by DA by ca. 30 %, caspase 3 activity increased by a factor of 3; all increases prevented by the presence of NAC.	PC12 cells exposed to DA: Increase in intracellular ROS by a factor of 2; completely reversed by NAC Quinoprotein formation increased by a factor of 3; completely prevented by the presence of NAC or GSH. Cell death increased from 3 % (control) to 37 % (DA). Reduced to 10 % in the presence of NAC.	PC12 cells and isolated rat brain mitochondria exposed to dopamine (100-400 µM). N-acetyl cysteine or GSH for protection were added at a concentration of 2.5 mM. In experiments including isolated mitochondria, NAC and GSH were added 2 h prior to DA. In experiments including PC12 cells, NAC and GSH were added 1 h prior DA. Isolated mitochondria were exposed to DA for 2 h; PC12 cells were expose to DA for 24 h.	Jana et al., 2011
Reduction of intracellular GSH by 50 % and of intramitochondrial GSH by 60 % leads to: Mitochondrial ROS increased by 30 % ATP levels reduced by 66 % Mitochondrial activity reduced by 66 % State 3 respiration reduced by 60 % Complex I activity inhibited by 60 %	Whole cell ROS increased by 30 %	PC12 cells with inducible knockdown of glutamyl cysteine synthetase (inhibition of GSH synthesis) by addition of 25 µg/ml doxycycline. Treatment for 24 h with doxycycline resulted in a GSH decline by ca. 50 %.	Jha et al., 2000
Reduction of GSH levels by ca. 50 % result in: Complex I inhibition by 40 %; completely reversed by DTT.	No cell toxicity under the applied conditions	N27 cells exposed to BSO (2.5 µM) for 7 days: Total glutathione was declined by ca. 50 % by this chronic treatment; absence of cell toxicity under these conditions. DTT for restoration of complex I activity was added at 1 mM.	Chinta et al., 2006

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Degeneration of dopaminergic neurons of the nigrostriatal pathway leads to Parkinsonian motor deficits (https://aopwiki.org/relationships/910)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Strong**	**Strong**

Evidence Supporting Applicability of this Relationship

Parkinonian disorders are generally recognized as progressive age-related human neurodegenerative diseases more prevalent in males. However, the anatomy and function of the nigrostriatal pathway is conserved across mammalian species (Barron et al. 2010) and no sex and species restrictions were evidenciated using the chemical stressors rotenone and MPTP. It should be noted that animal behaviour models can only be considered as surrogates of human motor disorders as occuring in Parkinson's disease.

How Does This Key Event Relationship Work

Degeneration of dopaminergic (DA) neuron terminals in the striatum and the degeneration of DA neurons in the substantia nigra pars compacts (SNpc) are the defining histopathological events observed in idiopathic, familial, and toxicant-evoked cases of Parkinson's Disease (PD) (Tolwani et al. 1999; Bove et al. 2012). The loss of nigrostriatal DA neurons leads to a decline in the levels of DA in the striatum (Koller et al. 1992). Striatal DA is involved in the modulation of extrapyramidal motor control circuits. A decline in striatal DA leads to an overactivation of the two principal basal ganglia output nuclei (GPi/STN). Therefore, the inhibitory GABAergic neurons that project to thalamo-cortical structures are overactivated and inhibit cortical pyramidal motor output performance. This inhibited output activity is responsible for key clinical symptoms of PD such as bradykinesia and rigor.

Weight of Evidence

Biological Plausibility

The mechanistic understanding of striatal DA and its regulatory role in the extrapyramidal motor control system is well established (Alexander et al. 1986; Penney et al. 1986; Albin et al. 1989; DeLong et al. 1990; Obeso et al. 2008; Blandini et al. 2000). The selective degeneration of DA neurons in the SNpc (and the subsequent decline in striatal DA levels) have been known to be linked to PD symptoms for more than 50 years (Ehringer et al. 1960). The reduction of DA in the striatum is characteristic for all etiologies of PD (idiopathic, familial, chronic manganese exposure) and related parkinsonian disorders (Bernheimer et al. 1973), and it is not observed in other neurodegenerative diseases, such as Alzheimer's or Huntington's Diseases (Reynolds et al. 1986). In more progressive stages of PD, not only a loss of DA neuronal terminals in the striatum, but also a degeneration of the entire DA neuron cell bodies in the substantia nigra pars compacta (SNpc) was detected (Leenders et al.

1986; Bernheimer et al. 1973). The different forms of PD exhibit variations in the degradation pattern of the SNpc DA neurons. In idiopathic PD, for example, the putamen is more severely affected than the caudate nucleus (Moratalla et al. 1992; Snow et al. 2000). All different PD forms however are characterized by the loss in striatal DA that is paralleled by impaired motor output (Bernheimer et al. 1973). Characteristic clinical symptoms of motor deficit (bradykinesia, tremor, or rigidity) of PD are observed when more than 80 % of striatal DA is depleted (Koller et al. 1992). These findings on the correlation of a decline in striatal DA levels as a consequence of SNpc DA neuronal degeneration with the onset of clinical PD symptoms in man provide the rationale for the current standard therapies that aim to supplement striatal DA, either by the application of L-DOPA, or by a pharmacological inhibition of the endogenous DA degradation-enzyme monoaminde oxidase B (MAO-B). These treatments result in an elevation of striatal DA that is correlated with an improvement of motor performance (Calne et al 1970). The success of these therapies in man as well as in experimental animal models clearly confirms the causal role of dopamine depletion for PD motor symptoms.

Empirical Support for Linkage

The experimental support linking the degeneration of DA neurons of nigrostriatal pathways with the manifestation of motor symptoms characteristics of parkinsonian disorders comes from human clinical observations as well as from primates, mice and rat in vivo models using DA neuron ablation by toxicants. The levels of striatal DA corrected with the onset of PD symptoms, and dopaminergic degeneration preceed the onset of motor symptoms. The exemplary animal studies selected here are based on the use of MPTP or rotenone. The efficacy of MPTP or rotenone treatment depends on the regimen applied (acute, subacute, chronic administration), the age of the animals, and the strains used. For the interpretation of the studies, it is important that in some animal models the initial depletion of DA is only partially explained by neurite degeneration. The other contributing factors are downregulation of TH, and depletion of DA from synaptic terminals. These effects recover after 1-2 weeks. This makes the time point of measurement important for the correlation of effects. Moreover, the mouse brain has a very high plasticity after damage, so that motor deficits can recover after several weeks although there is pronounced dopaminergic neuro degeneration.

Rat in vivo models

- *Rat/rotenone: Correlation between striatal DA, SNpc DA neurons, and motor deficits. Lewis rats exposed to systemic rotenone (3 mg/kg/ day i.p.) exhibited a loss of TH positive neurons in the SNpc by 45 %. Motor deficits were assessed by the postural instability test and by the rearing test. While 3 month old animals developed motor symptoms after 12 days of rotenone exposure, 7 month and 12 month old animals developed motor symptoms already after 6 days of exposure. Rotenone treatment elicited a progressive development of motor deficits that was reversible when treated with a DA agonist. Similar to that, the loss of rearing performance evoked by rotenone was reversed by the DA agonist apomorphine. Rotenone elicited terminal loss in the dorsolateral structures. While in the dorsolateral striatum, a significant loss of TH-positive neurites was detected, striatal cell bodies were spared from degeneration. Initial striatal DA levels (75 ng/ mg protein) dropped to 45 % following rotenone treatment (Cannon et al. 2009).*
- *Rat/6-OHDA: Destruction of nigrostriatal DA neurons. Unilateral injection of 6-OHDA into the dopaminergic nigrostriatal pathway leads to a preferential loss of DA neurons that is correlated with the onset of rotational motor deficits (Luthman et al. 1989; Perese et al. 1989; Przedborski et al. 1995).*
- *Rats/rotenone: Correlation between striatal dopamine and motor symptoms; partial reversibility by L-DOPA. Rats were exposed to 2.5 mg/kg rotenone, daily, for 48 days. Dopamine detected in the anterior striatum and posterior striatum was reduced by ca. 50 % after rotenone treatment. Rotenone treatment resulted in a significantly prolonged descent latency compared to control in the bar test and grid test. In the catalepsy test, descent latency dropped from 35 s of the controls to 5 s. In the grid test, a reduction from 30 s (control) down to 4 s (rotenone) was observed. The average distance travelled within 10 min by the animals was reduced from 37 m to 17 m in the rotenone group. Average number of rearings declined from 65 to 30; the time of inactive sitting of 270 s in controls was increased to 400 s in the rotenone group (Alam et al. 2004).*
- *Rat/rotenone: Correlation between striatal dopamine and motor symptoms. Rats were treated with rotenone either at doses of 1.5 mg/kg or 2.5 mg/kg over two months with daily i.p. injections. In the 2.5 mg/kg group, striatal DA levels dropped from 6400 pg/mg in the controls to 3500 pg/mg in the rotenone group. Rotenone treated animals showed an extended descent latency (5 to 50). In a vertical grid test, latency time increased from 9 s to 72 s (Alam et al. 2002).*
- *Rats/rotenone: Correlation between nigrostriatal TH intensity and motor symptoms. Rats were treated with different doses of rotenone for 21 days with daily i.v. or s.c. injections. In the 2.5 mg/kg group, TH intensity in the striatum dropped from 0.2 to 0.12. The average time to initiate a step increased from 5 s in the controls to 11 s in the rotenone group. Spontaneous rearing scores dropped from 80 % of the vehicle treated controls to 20 % in the rotenone group (Fleming et al. 2004).*
- *Rat/rotenone: In middle-aged rats exposed to rotenone (3 mg/kg/day for 6 days), a reduction of striatal DA levels and TH positive neurons by ca. 50 % correlated with impairments rearing performance and postural instability tests (Cannon et al. 2009).*
- *Rat/rotenone: In rats, exposed to rotenone (2.5 mg/kg/day), spontaneous locomotor activity was reduced*

by ca. 50 % after 1 week of rotenone treatment. This impaired motor performance was correlated with a loss of striatal DA fibers by 54 % and a loss of nigral DA neurons by 28.5 % (Höglinger et al. 2003).

Mouse in vivo models

- *Mouse/MPTP: In mice exposed to MPTP in combination with probenecid, both a chronic treatment scheme (MPTP 25 mg/kg, in 3.5 day intervals for 5 weeks) as well as a subacute treatment scheme (25 mg/kg, 1x per day for 5 days) resulted in a deletion of striatal DA that was directly correlated with impairments in motor symptoms (Petroske et al. 2001).*
- *Mouse/MPTP: In a mouse model exposed to MPTP at 15 day intervals (36 mg/kg), lower rotarod performance was observed after the fourth injection. The decline in motor performance was correlated with the decline in TH-immunoreactivity in the striatum (r2 = 0.87) (Rozas et al. 1998).*
- *Mouse/D2 receptor knockout. Mice deficient in D2 receptors displayed akinesia, bradykinesia and a reduction in spontaneous movement (Baik et al. 1995).*

Monkey in vivo models

- *Monkey/MPTP: Correlation between striatal DA, SNpc DA neuron number and PD symptoms. Macaca exposed to MPTP (i.v) (0.2 mg/kg, daily) display signs of PD at day 15, including motor abnormalities. The transition between the presymptomatic and symptomatic period occurred between day 12 and day 15 of MPTP exposure. At day 15, TH neurons in the SNpc were reduced by 50%, DAT binding autoradiography studies revealed a decline in binding also by 50% at day 15. Compared with control values of 150 pg/µg protein, the DA content of the caudate nucleus dropped to values < 10 pg/µg protein at day 15. In the putamen, DA levels dropped from 175 pg/µg protein to 20 pg/µg protein at day 15 (Bezard et al. 2001).*
- *Monkey/MPTP: Correlation between striatal DA, SNpc DA neurons, and PD symptoms. Monkeys display a motor symptom pattern similar to that observed in humans. In order to optimize a MPTP intoxication protocol that allows a gradual development of nigral lesion, different states of PD symptom severity were defined and correlated with the amount of striatal DA and the number of TH-positive neurons in the SNpc. Asymptomatic monkeys displayed a reduction in striatal DA by 30 %, a neuronal loss in the SNpc by 40 %, and a decline in striatal expression of TH, DAT and VMAT2 by 50-60 %. Monkeys that recovered from early PD symptoms displayed a reduction of striatal DA of 50 %, a loss of TH neurons in the SNpc and a loss of DAT and VMAT2 expression up to 60 %. In animals with moderate PD symptoms, striatal DA levels as well as TH positive neurons and DAT and VMAT 2 expression were reduced by 70-80 %. Animals with severe PD symptoms displayed remaining levels of striatal DA and SNpc expression of TH, DAT and VMAT2 of around 20 % compared to untreated controls (Blesa et al. 2012).*
- *Monkey/MPTP: The established model of basal ganglia wiring received ample experimental support in recent years. For instance, an increase in the inhibitory output by GPi/STN has been observed in MPTP treated monkeys, similar to the situation in idiopathic PD patients. These findings were corroborated by observations indicating an elevated mitochondrial activity and an elevated firing rate of the inhibitory output nuclei detected on the level of individual neurons (Mitchell et al. 1989; Filion et al. 1991). Lesions in the output ganglia of monkeys lead to a reduction in the output and to an improvement in motor control (Bergman et al. 1990; Aziz et al. 1991). In analogy to these lesion experiments, deep brain stimulation of these regions results in a profound improvement of motor performance in PD patients (Limousin et al. 1999; Ceballos-Baumann et al. 1994).*

Human PD

- *Human PD: Association of PD phenotype with impaired striatal DA. In the brains of human PD patients, a significant decrease of striatal DA was observed (Lloyd et al. 1975). In the caudate nucleus, levels of DA dropped from control values of 4 µg/g tissue to levels of 0.2 µg/g. In the putamen, control values were in the range of 5 µg/g and 0.14 µg/g in the PD patient group. The levels of DA in the striata of DA patients that received L-DOPA treatment was 9-15 times higher compared with non-treated PD cases.*
- *Human PD: Correlation between striatal DA loss and degeneration of DA neurons in the SNpc. Examinations of the brains of PD patients revealed morphological damage in the SNpc, accompanied by the degeneration of DA neurons (Earle et al. 1968).*
- *Human: Association of striatal DA levels and motor performance. In order to substitute degenerated DA neurons in the SNpc, human fetal tissue from the ventral mesencephalon was transplanted to the caudate and putamen in idiopathic cases PD as well as in patients that developed PD-related motor deficits as a consequence to MPTP intoxication. Transplanted cells led to a reinnervation of the striatum with DA projections (Widner et al. 1992; Kordower et al. 1995, 1998). In these case studies, patients demonstrated a sustained improvement in motor function (decline in rigidity score by more than 80 %).*
- *Human PD: correlation between nigrostriatal DA neuron content and motor symptoms. Imaging of DAT was performed by the use of 123I-FP-CIT SPECT (single photon emission computed tomography). Clinical PD severity was determined by using the Unified Parkinsons Disease Rating Score (UPDRS). In PD patients, DAT binding in the striatum, caudate, and putamen correlated with disease severity and duration of disease (Benamer et al. 2000).*

- *Human PD: correlation between 18F-dopa uptake measured by PET and the onset of motor symptoms detected according the UPDRS. 18F-dopa influx rate constants (Ki/min) were reduced in the midbrain from 0.008 to 0.006, in the right putamen from 0.017 to 0.0036, and in the left putamen from 0.017 to 0.005 (Rakshi et al. 1999).*
- *Human PD: correlation between putamen influx rate (Ki/min). Ki (control): 0.0123; asymptomatic PD (no observable motor deficits): 0.0099; symptomatic PD (clinically evident motor deficits): 0.007. Mean UPDRS value was 15.1 7.5. A correlation coefficient of -0.41 was detected between motor UPDRS and putamen influx (Ki) (Morrish et al. 1995).*
- *Human PD: Correlation of the degree of monoaminergic degeneration in early PD with motor symptoms assed by the UPDRS and the Hoehn and Yahr Stage scale. For PET imaging, 18F-9-fluoropropyl-dihydrotetrabenzazine that targets VMAT2 was used. Uptake of the tracer was reduced by 20-36 % in the caudate, by 45-80 % in the putamen, and by 31 % in the substantia nigra. This correlated with a total UPDRS value of 12.1 7.1 in the PD group, respectively with a HY value of 1.0 0.1 in the PD group compared to controls (Lin et al. 2014).*
- *Human PD: Correlation between the decline in 18F-dopa rate constant (Ki) and the onset of motor deficits. The 18F-dopa rate constant Ki was reduced in the caudate nucleus (0.011 down to 0.0043) and inversely correlated with an increase in the UPDRS from 11.9 5.2 to 50 11.6 (Broussolle et al. 1999).*
- *Human PD: Correlation between striatal DAT binding measured by the use of 123I-CIT SPECT and motor deficits. A correlation coefficient between 123I-CIT binding and UPDRS motor scale of -0.56 was detected. A correlation coefficient of -0.64 between 123I-CIT binding and Hoehn and Yahr stage scale was detected. Motor symptoms in the clinically less affected body side show a closer correlation with striatal DAT binding (Pirker et al. 2003).*
- *Human PD: Correlation between the reduction in the putamen uptake of 18F-CFT and the severity of PD motor symptoms. 18F-CFT uptale was reduced to 18 % in the putamen, to 28% in the anterior putamen, and to 51 % in the caudate nucleus (Rinne et al. 1999).*
- *Human PD: Reduction in 123I-CIT binding in the putamen by 65 % correlated with a mean UPDRS score of 27.1 (Tissingh et al. 1998).*
- *Association between striatal DA and motor performance. Application of L-DOPA leads to a substitution of DA in the striatum and improves motor performance. (Boraud et al. 1998; Gilmour et al. 2011; Heimer et al. 2002; Papa et al. 1999; Hutchonson et al. 1997; Levy et al. 2001).*

Uncertainties or Inconsistencies

- *Motor abnormalities observed in PD display large interindividual variations.*
- *The model of striatal DA loss and its influence on motor output ganglia does not allow to explain specific motor abnormalities observed in PD (e.g. resting tremor vs bradykinesia) (Obeso et al. 2000). Other neurotransmitters (Ach) may play additional roles*

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- *There are some reports indicating that in subacute rotenone or MPTP models (non-human primates), a significant, sometimes complete, recovery of motor deficits can be observed after termination of toxicant treatment. While the transient loss of striatal DA can be explained by an excessive release of DA under acute toxicant treatment, the reported losses of TH-positive neurons in the SNpc and their corresponding nerve terminals in the striatum are currently not explained (Petroske et al. 2001).*
- *In MPTP treated baboons, the ventral region of the pars compacta was observed to be more severely degenerated that the dorsal region. This pattern is similar to the degeneration pattern in idiopathic PD in humans. These observations indicate that two subpopulations of nigrostriatal DA neurons with different vulnerabilities might exist (Varastet et al. 1994).*
- *According to the classical model of basal ganglia organization, DA is assumed to have a dichotomous effect on neurons belonging either to the direct or indirect pathway. More recent evidence however rather indicates that D1 and D2 receptors are expressed on most striatal neurons in parallel (Aizman et al. 2000).*

Quantitative Understanding of the Linkage

An example of quantitative analysis is reported in the table below. The analysis of the empirical data produced with the chemical toxicants supports a strong response- response relationship between the KE up and the KE down which also indicative of the temporal progression and relationship between the degeneration of striatal terminals of DA neurons, loss of DA neurons in the SNpc and the occurrence and severity of the motor deficits. This is also quantitatively supported by studies conducted in human PD patients.

Upstream key event (KE 4)	***Downstream key event (AO)***	***References***	***Comments***
Rat models			

45 % loss of TH-positive SNpc neurons in 7 month old rats, ca. 40 % loss in 12 month old rats *Striatal DA reduced from 90 ng/mg (control) down to 45 ng/mg* *TH pos. neuron number* *18000 (control)* *10000 (rotenone)*	*Bradykinesia, postural instability, rigidity observed in 50 % of cases:* *3 month old rats: after 12 days of rotenone* *7 + 12 month old rats. After 6 days of rotenone* *Postural instability test:* *Distance required for the animal to regain postural stability:* *3.5 cm (control)* *5 cm (rotenone)* *Rearing test (rears/ 5 min):* *10 (control* *3 (rotenone)* *Loss of rearing performance evoked by rotenone was reversed by the DA agonist Apomorphine in 3 month old rats*	*Cannon et al. 2009*	*Lewis rats + rotenone (3 mg/kg/day, i.p. daily)*
Dopamine in the anterior and posterior striatum reduced by ca. 50 %.	*Catalepsy test: decline from 35 s to 5 s.* *Grid test: decline from 30 s to 4 s* *Distance travelled in 10 min: reduction from 37 m to 17 m.* *Number of rearings: decline from 65 to 30.* *Inactivity time increased from 270 s to 400 s.* *Partial reversibility by L-DOPA treatment:* *L-DOPA: number of rearings increased from 16 to 30.* *L-DOPA: inactivity time reduced from 450 s to 360 s.* *L-DOPA: increase in the distance travelled from 12 to 16 m.*	*Alam et al. 2004*	*Rats + rotenone (2.5 mg/kg) daily over the course of 48 days.*
TH staining intensity reduced from 0.2 to 0.12	*Rearing scores reduced from 80 % (vehicle controls) to 20 % (rotenone group).* *Increase in the average time to initiate a step from 5 s to 11 s.*	*Fleming et al. 2004*	*Rats + rotenone 2.5 mg/kg for 21 days i.v. or s.c.*
Loss of striatal DA fibers by 54 % *Loss of DA neurons by 28.5 %*	*Spontaneous locomotor activity after 1 week* *100 % (control)* *55 % (rotenone)*	*Höglinger et al. 2003*	*Rats + rotenone (2.5 mg/kg/day for 28 days*
Mouse models			

Subacute model: *Striatal DA dropped from 11 ng/mg (control) to 2.5 ng/mg (MPTP) after 3 days.* *3H-DA striatal uptake reduced from 2.9 pmol/mg (control) to 1.3 pmol/mg after 3 days of MPTP.* *Total nigrostriatal TH cell count was not affected.* *Chronic model:* *Striatal DA content reduced from 13 ng/ml down to 0.5 ng/ml at 1 week after MPTP treatment.* *3H-DA uptake in the striatum reduced from 3 pmol/mg to 1 pmol/mg 1 week after start of MPTP treatment.* *TH staining in the nigrostriatal system reduced by ca. 50 % 1 week after initiation of MPTP treatment.*	*Subacute model:* *Rotarod performance reduced from 1800 AUC (control) down to 1500 AUC (MPTP).* *Chronic model:* *Rotarod performance reduced from 1800 AUC (control) to 1250 AUC (1 week after initiation of MPTP treatment)*	*Petroske et al. 2001*	*Mouse + MPTP* *Subacute model:* *25 mg/kg MPTP 1x days for 5 days* *Chronic model:* *MPTP (25 mg/kg + 250 mg/kg probenizid) in 3.5 day intervals for maximal 5 weeks*
Reduction in TH staining intensity of at least 50 % required for detectable influence on motor performance. *TH density in the nigrostriatal system correlated with the decline of rotarod performance (r2 = 0.87)*	*Rotarod performance reduced from 1250 AUC to 200 AUC* *Time on rod at a speed of 20 rpm:* *125 s in controls, 25 s in MPTP animals*	*Rozas et al. 1998*	*Mouse + MPTP*
Monkey models			
Approx. 50 % loss of TH positive neurons in the SNpc. DA content in the caudate nucleu reduced to < 10 %; DA content of the putamen ca. 10 % compared with control	*Mean duration in the bradykinesia test increased from 3 sec. (day 0) to 19 sec. at day 15*	*Bezard et al. 2001*	*Macaca + MPTP i.v. 0.2 mg/kg daily for 15 days*
Human			
18F-dopa influx rate constants (Ki) *Midbrain:* *Control: 0.008* *Early PD: 0.008* *Adv. PD: 0.006* *Right putamen:* *Control: 0.017* *Early PD: 0.006* *Adv. PD: 0.0036* *Left putamen:* *Control: 0.017* *Early PD: 0.0096* *Adv. PD: 0.005*	*Early PD:* *UPDRS: 9 +/- 3* *Adv. PD:* *UPDRS: 41 +/- 15*	*Rakshi et al. 1999*	*Human PD patients*
Putamen influx (Ki/min) detected by 18F-dopa *control: 0.0123* *asympt. PD: 0.0099* *symptom. PD: 0.007*	*Symptom. PD patients: mean UPDRS: 15.1 +/- 7.5* *Correlation between total UPDRS and putamen Ki:* *r = -0.41*	*Morrish et al. 1995*	*Human PD*

Uptake of 18F-DTBZ (VMAT2 tracer) reduced by: *20-36 % (caudate)* *45-80 % (putamen)* *31 % (SN)*	*UPDRS total: 12.1 +/- 7.1* *Hoehn and Yahr : 1.0 +/- 0.1*	*Lin et al. 2014*	*Human PD*
Caudate nucleus Ki/min *Control: 0.011* *PD group 3: 0.0067* *Putamen Ki/min* *Control: 0.011* *PD group 3: 0.0043*	*UPDRS: 50 +/- 11.6 in PD group 3*	*Broussolle et al. 1999*	*Human PD*
Reduction in 18F-CFT uptake in the posterior putamen (by 18 %); in the anterior putamen (by 28 %); in the caudate nucleus (by 51 %)	*Correlation between total motor score of the UPDRS and 18F-CFT uptake:* *Posterior putamen:* *r = -0.62* *Anterior putamen:* *r = -0.64* *Caudate nucleus:* *r = -0.62*	*Rinne et al. 1999*	*Human PD*
123I-CIT SPECT values in controls and PD cases with a Hoehn and Yahr rating of 2-2.5: *Putamen (ipsilateral):* *Control: 6.13* *PD: 1.84* *Caudate (ipsilateral):* *Control: 6.93* *PD: 3.66* *Striatum* *(ipsilateral):* *Control: 6.28* *PD: 2.33*	*Correlation coefficient between striatal 123I-CIT binding and:* *Str. (ipsilateral) and Bradykinesia:* *r = -0.61* *Str. (ipsilateral) and Rigidity:* *r = -0.46* *Str. (ipsilateral) and UPDRS:* *r = -0.79*	*Tissingh et al. 1998*	*Human PD*
Binding ration striatum/cerebellum detected by 123I-CIT / SPECT *Control:* *8.71 +/- 1.54* *PD:* *4.49 +/- 1.86*	*Correlation between 123I-CIT binding to DAT and PD motor symptoms rated according to the Hoehn and Yahr scale:* *r = -0.75* *Correlation according to the UPDRS:* *r = -0.49*	*Asenbaum et al. 1997*	*Human PD*
Uptake of 123I-CIT in the putamen reduced to 54 %; uptake into the caudate nucleus reduced to 65 %	*Correlation between CIT uptake in the putamen and Hoehn and Yahr stage:* *r = -0. 79*	*Rinne et al. 1995*	*Human PD*

Decline in nigrostriatal DAT assed by 123I-CIT SPECT in PD patients	*Correlation coefficients for 123I-CIT uptake in the striatum and:* *UPDRS: r =-0.54* *Bradykinesia: r = -0.5* *Rigidity: r = -0.27* *Tremor: r = -0.3* *Correlation coefficients for 123I-CIT uptake in the caudate and:* *UPDRS: r =-0.5* *Bradykinesia: r = -0.43* *Rigidity: r = -0.27* *Tremor: r = -0.26* *Correlation coefficients for 123I-CIT uptake in the putamen and:* *UPDRS: r =-0.57* *Bradykinesia: r = -0.53* *Rigidity: r = -0.29* *Tremor: r = -0.37*	*Benamer et al. 2000*	*Human PD*

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Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I) leads to Inhibition, NADH-ubiquinone oxidoreductase (complex I) (https://aopwiki.org/relationships/933)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Strong**	**Weak**

Evidence Supporting Applicability of this Relationship

The CI is well-conserved across species from lower organism to mammals. The central subunits of CI harboring the bioenergetic core functions are conserved from bacteria to humans. CI from bacteria and from mitochondria of Yarrowia lipolytica, a yeast genetic model for the study of eukaryotic CI (Kerscher et al., 2002) was analyzed by x-ray crystallography (Zickermann et al., 2015). However, the affinity of various chemicals to cause partial or total inhibition of CI activity across species is not well studied (except rotenone).

How Does This Key Event Relationship Work

It is well documented that binding of an inhibitor to CI inhibits its activity (see MIE). Naturally occurring and synthetic CI inhibitors have been shown to inhibit the catalytic activity of CI, leading to partial or total inhibition of its activity in a dose response manner (Degli Esposti and Ghelli, 1994; Ichimaru et al. 2008; Barrientos and Moraes, 1999; Betarbet et al., 2000). Indeed, binding of inhibitors stops the electron flow from CI to ubiquinone. Therefore, the Fe-S clusters of CI become highly reduced and no further electrons can be transferred from NADH to CI. This leads to the inhibition of the NADH oxido-reductase function, i.e. CI inhibition.

Weight of Evidence

Biological Plausibility
The weight of evidence supporting the relationship between binding of an inhibitor to NADH-ubiquinone oxidoreducatse and its inhibition is strong.

Biological Plausibility

There is an extensive understanding of the functional relationship between binding of an inhibitor to NADH-ubiquinone oxidoreductase (CI) and its inhibition. As the first entry complex of mitochondrial respiratory chain, CI oxidizes NADH and transfers electrons via a flavin mononucleotide cofactor and several Fe-S complexes to ubiquinone. The electron flow is coupled to the translocation of protons from the matrix to the intermembrane space. This helps to establish the electrochemical gradient that is used to fuel ATP synthesis (Greenamyre et al., 2001). If an inhibitor binds to CI, the electron transfer is blocked. This compromises ATP synthesis and maintenance of Δψm, leading to mitochondrial dysfunction. As CI exerts a higher control over oxidative phosphorylation in synaptic mitochondria than in non-synaptic mitochondria in the brain (Davey and Clark, 1996), specific functional defects observed in PD may be explained. It is well documented that CI inhibition is one of the main sites at which electron leakage to oxygen occurs. This results in a production of ROS, such as superoxide (Efremov and Sazanow, 2011) and hydrogen peroxide, which are main contributors to oxidative stress (Greenamyre et al., 2001).

Empirical Support for Linkage
A variety of studies show a significant correlation between binding of an inhibitor to CI and its inhibition, usually measured by the decreased mitochondrial respiration. Different classes of CI inhibitors, such as rotenone, MPP+, piericidin A, acetogenins, pyridaben, and various piperazin derivatives (Ichimaru et al. 2008) have been shown to bind to the ubiquitin site of CI, leading to a partial or total inhibition of oxidoreductase activity in a dose response manner (Grivennikova et al., 1997; Barrientos and Moraes, 1999; Betarbet et al., 2000). The reduction of CI activity is well documented in a variety of studies using isolated mitochondria or cells, as well as in in vivo experiments and in human post mortem PD brains. Usually it is measured by assays described in 2nd Key Event Relationship (KER): Inhibition of complex I leads to mitochondrial dysfunction. It has been shown that binding of rotenone to CI (e.g. Betarbet et al., 2000, Greenamyre et al., 2001) or MPP+ (e.g. Krug et al., 2014; Langston, 1996) can reproduce the anatomical, neurochemical, behavioural and neuropathological features of PD. Therefore, the empirical support for this KER will be mainly based on the experiments performed after exposure to rotenone or MPP+.

- The binding of rotenone to CI resulted in time- and dose-dependent inhibition of CI activity measured in sub-mitochondrial particles. The kinetics of the active CI inhibition was determined after exposure to

rotenone at 20, 30 and 40 nM at different times of exposure (30 sec, 1 min or 2 min) (Grivennikova et al., 1997). This study suggests that two rotenone binding sites exist in CI: one affecting NADH oxidation by ubiquinone and the other one operating in ubiquinol-NAD+ reductase action.

- Partial inhibition of CI produces a mild, late-onset mitochondrial damage. The threshold effect seen in brain mitochondria (25–50% decrease in activity) may not directly impact ATP levels or Δψm but could have long-term deleterious effects triggered by oxidative stress, as it has been shown that an electron leak upstream of the rotenone binding site in CI leads to ROS production (Greenamyre et al., 2001).
- Exposure of rats to rotenone (2 days, 2 mg/kg) produced free brain rotenone concentration of 20–30 nM and resulted in 73% inhibition of specific binding to CI of [3H] dihydrorotenone (Betarbet et al., 2000). However, oximetry analysis indicated that in brain mitochondria (but not liver mitochondria) this rotenone concentration (30 nM maximum) was insufficient to inhibit glutamate (CI substrate)-supported respiration (Betarbet et al., 2000) suggesting that this rotenone concentration did not alter mitochondrial oxygen consumption in isolated brain mitochondria.
- Rotenone has been reported to be a specific and potent mitochondrial CI inhibitor with IC50 values from 0.1 nM to 100 nM depending on the system and methods used (Lambert and Brand, 2004; Ichimaru et al., 2008; Chinopoulos and Vizi, 2001; Beretta et al., 2006).
- Mesencephalic cultures prepared from C57/BL6 mice and treated with 5, or 10 nM rotenone for 24 h inhibited CI activity by 11% or 33%, respectively (Choi et al., 2008).
- The inhibition of CI was studied in the human osteosarcoma-derived cell line (143B) after the exposure to rotenone or using a genetic model (40% loss of CI activity in human xenomitochondrial cybrids (HXC) lines). Different degrees of CI inhibition were quantitatively correlated with levels of decreased cellular respiration (Barrientos and Moraes, 1999). Only when CI was inhibited by 35-40% (< 5 nM rotenone), cell respiration decreased linearly until 30% of the normal rate. Increasing concentrations of rotenone produced further but slower decrease in CI activity and cell respiration (Fig. 1). Cells with the complete rotenone-induced CI inhibition still maintain a cell respiration rate of approximately 20% because of an electron flow through complex II. At high concentrations (5–6-fold higher than the concentration necessary for 100% CI inhibition), rotenone showed a secondary, toxic effect at the level of microtubule assembly (Barrientos and Moraes 1999).
- Bovine sub-mitochondrial particles were used to test rotenone affinity binding at 20 nM. This concentration of rotenone reduced the NADH oxidation rate by approximately 50% (Okun et al., 1999.
- MPP+ (an active metabolite of MPTP) is an inhibitor of CI (Nicklas et al., 1987; Mizuno et al, 1989; Sayre et al., 1986). Inhibition of the mitochondrial CI by MPP+ supresses aerobic glycolysis and ATP production (Book chapter in Cheville 1994).
- MPP+ binds loosely to CI and causes reversible inhibition of its activity: approximately 40% inhibition was observed at 10 mM concentration within 15 min of incubation. However, prolonged incubation (> 15min) produces up to 78% of irreversible inhibition of CI (Cleeter et al., 1992).

Human studies

- There are many studies that show impaired catalytic activity of CI in multiple PD post-mortem brain tissues. For example (Parker and Swerdlow, 1998), five PD brains were used to measure activities of complexes I, III, IV, and of complexes I/III together (NADH: cytochrome c reductase). These measurements were performed in purified frontal cortex mitochondria and revealed a significant loss of CI activity in these PD samples as compared to controls.
- Human data indicate that impairment of CI activity may contribute to the pathogenic processes of PD (for example, Greenamyre et al., 2001; Schapira et al., 1989; Shults, 2004).

Uncertainties or Inconsistencies

It is not clear the number of subunits constituting CI in mammals, as according to the existing literature different numbers are cited (between 41-46) (Vogel et al., 2007a; Hassinen, 2007). The majority of data claims that mammalian CI is composed of 46 (Greenamyre et al., 2001; Hassinen, 2007) or 45 subunits (Vogel et al., 2007a). It is not sure whether there may exist tissue-specific subunits of CI isoforms (Fearnley et al., 2001). It is unclear, which subunit(s) bind rotenone or other inhibitors of CI. Additionally, it is not clear whether CI has other uncharacterized functions, taking into consideration its size and complexity (43-46 subunits vs. 11 subunits of complex III or 13 subunits of complex IV) (Greenamyre et al., 2001). There is no strict linear relationship between inhibitor binding and reduced mitochondrial function. Low doses of rotenone that inhibit CI activity partially do not alter mitochondrial oxygen consumption. Therefore, bioenergetic defects can not account alone for rotenone-induced neurodegeneration. Instead, under such conditions, rotenone neurotoxicity may result from oxidative stress (Betarbet et al., 2000). Few studies used human brain cells/human brain mitochondria. Therefore, full quantitative data for humans are not available.

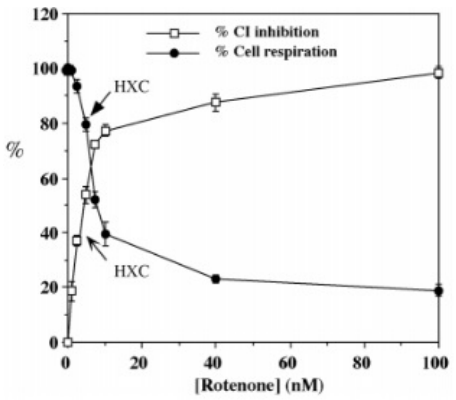
Quantitative Understanding of the Linkage

The kinetics of binding and CI inhibition by rotenone has been quantitatively evaluated in a dose-dependent manner using the sub-mitochondrial particles (Grivennikova et al., 1997). The consequences of CI inhibition were quantitatively measured by a variety of assays that are used to study mitochondrial dysfunction (see Key

Event Relationship (KER): Inhibition of Complex I leads to mitochondrial dysfunction). There are also many in vitro and in vivo studies combining the quantification of CI inhibition and DA cell death (e.g. Choi et al., 2008, Betarbet et al., 2000, see KER Mitochondrial dysfunction induces degeneration of nigrostriatal pathway).

The binding of different classes of inhibitors (e.g., pesticides, drugs and other toxins) to CI has been determined quantitatively and I50, and KI values are available. Potency relative to that of rotenone has been determined under the same conditions in beef mitochondria or submitochondrial particles using the ratio of the KI values, when they were available (Degli Esposti, 1998; Okun et al., 1999). Rotenone I50 value is defined as 20 nM (Okun et al., 1999).

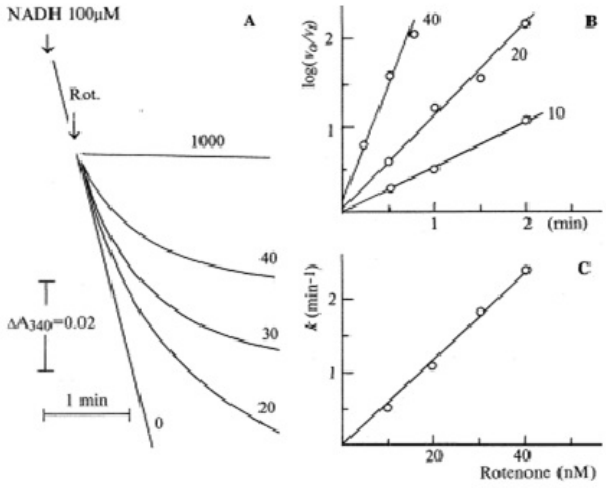
Example of a quantitative evaluation of concentration-dependent CI inhibition by rotenone (Fig. 1 from Barrientos and Moraes, 1999).



(https://aopwiki.org/wiki/index.php/File:KER_1Fig._1.jpg)

Fig. 1. Fig.1. Effect of CI (NADH decylubiquinone reductase) inhibition on endogenous cell respiration. Cells were treated with different concentrations of rotenone for 4 h before measuring cell respiration in whole cells and CI activity in isolated mitochondria. Complete CI inhibition was achieved with 100 nM rotenone. The cell respiration was inhibited also in a dose-dependent manner but showed different inhibition kinetics and a saturation threshold. For comparison, the genetically-altered cell line HXC had an approximately 40% CI reduced activity and an approximately 80% residual cell respiration. HXC, human xenomitochondrial cybrids.

Time- and concentration-relationship of NADH oxidase inhibition by rotenone (Fig. 2. from Grivennikova et al., 1997).



(https://aopwiki.org/wiki/index.php/File:KER1_Fig._2.jpg)

Fig. 2. Panel A and B: Time- and concentration-relationship of NADH oxidase inhibition by rotenone. The numbers on the curves indicate the final concentrations of rotenone (0, 20, 30, 40, 1000 nM). In Panel B: vo, zero-order rate of NADH oxidation in the absence of rotenone; vt, the `instant' values of the rates approximated within 10 s time intervals. Panel C: The dependence of first-order inhibition rate constant on the concentration of rotenone (for further description see Fig. 1 in Grivennikova et al., 1997).

Quantitative evaluation of the 1st KER: Binding of inhibitor to NADH-ubiquinone oxidoreductase (MIE; KE upstream) leads to its inhibition (KE downstream)

'MIE (KE upstream)' *Binding of inhibitor to NADH-ubiquinone oxidoreductase (nM)*	***'KE (downstream)'*** Inhibition of CI (%, approximately)	***'Comments'*** (in vivo, in vitro or human studies)	***'References'***
Administration of rotenone at 2 mg/kg per day for 2 days resulted in free rotenone concentration of 20–30 nM in the brain.	75%	DA neuronal cell death determined after rotenone administration at 1 to 12 mg/kg per day, Sprague Dawley and Lewis rats infused continuously by jugular vein, 7days up to 5 weeks	Betarbet et al., 2000
20 nM rotenone Direct binding studies using bovine and Musca domestica sub-mitochondrial particles	50%	Binding studies that defined the I50 and Kd values for three classes of CI inhibitors (12 chemicals) including rotenone.	Okun et al., 1999
Human skin fibroblasts exposed to 100 nM Rotenone for 72 hr	20%	In the same experiment mitochondria morphology, motility was also evaluated.	Koopman et al., 2007
0-2.5 nM Rotenone 5/10 nM Rotenone Mesencephalic neurons were cultured from E14 C57/BL6 mouse embryos for 6 days and then treated with rotenone for 24 hr	No effect 11% and33%, respectively	Treatments with 5 or 10 nM rotenone killed 50% or 75% DA neurons respectively.	Choi et al., 2008
1-2.5-5-7.5-10-20 nM 1-10-20-80 nM	10-20-35-50-65-80 % 5- 75 %	In this study time course of the active and deactivated enzymes inhibition by rotenone and Piericidin A is study in a dose-dependent manner. Binding studies in sub-mitochondrial particles prepared from bovine heart after 20 min of exposure to rotenone.	Grivennikova et al., 1997
5-10 nM 20 nM 40 nM 100 nM 143B Cells (human osteo-sarcoma), exposed for 4 hrs to rotenone	55-78 % 80% 87% 100%	In the same study similar experiments were performed using HXC cell line (see Fig. 1 above).	Barrientos and Moraes 1999

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Inhibition, NADH-ubiquinone oxidoreductase (complex I) leads to N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/relationships/934)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	**directly leads to**	**Strong**	**Moderate**

Evidence Supporting Applicability of this Relationship

Mitochondrial CI in eukaryotes has highly conserved subunit composition based on protein databases (Cardol, 2011). The characterization of induced mitochondrial dysfunction phenotypes in zebrafish was studied in the presence of CI and CII inhibitors (Pinho et al., 2013). Exposure of Caenorhabditis elegans (C. elegans) to rotenone, reduced bioluminescence (an assay for mitochondrial dysfunction) after both relatively short (2 hr) and longer exposures (24 hr) to a range of concentrations. A sharp decline in bioluminescence (maximal inhibition) relative to controls occurred at the lowest rotenone concentration of 2.5 µM. This decline in bioluminescence was consistent with reduced cellular ATP (Lagido et al., 2015). The results obtained from C. elegans exposed to rotenone suggested that chronic exposure to low concentration (2 or 4 µM) caused mitochondrial damage through persistent suppression of mitochondrial biogenesis and mitochondrial gene expression leading to mitochondrial dysfunction that contributed to DA neuron degeneration (Zhou et al., 2013).

Drosophila melanogaster has been proven suitable to study signaling pathways implicated in the regulation of mitochondrial function and integrity, such as the PINK1/parkin pathway (controlling mitochondrial integrity and maintenance), DJ-1 and Omi/HtrA2 genes (associated with the regulation of mitochondrial functionality). Notably, PINK1, PARKIN, and DJ-1 genes are associated with recessive forms of PD (Guo, 2012). Drosophila flies lacking DJ-1 result to be viable, but show an increased sensitivity to oxidative stress induced upon rotenone or Paraquat (an herbicide inducer of CI-dependent ROS) feeding (Menzies et al. 2005; Meulener et al. 2005; Meulener et al. 2006). Moreover, it has been reported in Drosophila that inhibition of CI by mean of sublethal chronic exposure to rotenone (<750 µM) via the feeding medium caused a selective loss of DA neurons in all of the brain regions and locomotor impairments, while L-dopa (3,4-dihydroxy-L-phenylalanine) rescued the behavioral deficits (but not neuronal death) (Coulom and Birman, 2004). MPTP causes Parkinsonism in primates including humans. However, rodents (rats) are much less susceptible to MPTP+ but are fully susceptible to MPP+ (due to the differences in toxicokineticks). In all species, CI inhibition leads to mitochondrial dysfunction. Mitochondrial dysfunction is an universal event occurring in cells of any species (Farooqui and Farooqui, 2012).

How Does This Key Event Relationship Work

Inhibited CI is unable to pass off its electron to ubiquinone and it cannot translocate protons across the mitochondrial inner membrane. This creates a back-up of NADH within the mitochondrial matrix (Brown and Borutaite, 2004). This leads to an arrest of the citric acid cycle and a failure to build a proton gradient (mitochondrial membrane potential, Δψm) across the inner membrane. This results in impaired ATP production. In addition, the direct transfer of electrons from CI to oxygen is increased. This leads to oxidative stress as ROS (e.g. superoxide, hydrogen peroxide) are produced, which can damage DNA, proteins, lipids and other cell components and function (Sanders et al., 2014).

Weight of Evidence

The weight of evidence supporting the relationship between inhibition of CI and mitochondrial dysfunction is strong. The mechanisms behind this KER are partially understood and well documented based on in vitro as well as in vivo experiments (e.g., Sanders et al., 2014), complemented by data from human post-mortem PD brain evaluations (Parker et al., 1989; Greenamyre et al., 2001; Sherer et al., 2003; Schapira et al., 1989).

Biological Plausibility

The biological plausibility that inhibition of CI activity triggers mitochondrial dysfunction is strong. It is well understood, how the inhibition of CI can lead to mitochondrial dysfunction as measured by: a) decreased oxygen consumption, b) decrease or loss of ATP production, c) decrease of Δψm, d) the loss of mitochondrial protein import and protein biosynthesis, e) reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, f) elevated levels of ROS, g) the loss of mitochondrial motility, causing a failure of mitochondria to re-localize to sites of increased energy demands (such as synapses), h) destruction of the mitochondrial network, i) increased mitochondrial uptake of Ca2+ causing mitochondrial Ca2+ overload (Graier et al., 2007) and opening of mitochondrial PTP, (j) rupture of the mitochondrial inner and outer membranes, leading to release of mitochondrial pro-death factors, including cytochrome c, AIF and endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013). These pathological mechanisms are extremely well studied.

Empirical Support for Linkage

Many studies show that the pathophysiological consequences of a partial or total CI inhibition are linked to mitochondrial dysfunction. In many of these experiments the cellular damage caused by mitochondrial

dysfunction is reduced (or entirely prevented) by treatment with antioxidants. Different degrees of CI inhibition by rotenone have been studied in the human osteosarcoma-derived cell line (143B). A quantitative correlation between increasing inhibition of CI and mitochondrial dysfunction (as shown by inhibition of mitochondrial respiration, reduced ATP production, increased ROS release and lipid peroxidation, as well as decreased Δψm) was established (Fig. 1 and Table 1 based on Barrientos and Moraes, 1999). Based on the existing literature it is suggested that rotenone exerts toxicity via oxidative stress, rather than via decrease of ATP synthesis (bioenergetics effects).

A few examples illustrating mitochondrial damage and oxidative stress in animal model of PD and human cells induced by:

Rotenone

- Rotenone administered subcutaneously for 5 weeks (2.5 mg/kg/d) caused a selective increase (by ~2 folds) in oxidative damage in the striatum, as compared to the hippocampus and cortex, accompanied by massive degeneration of DA neurons (~80% decrease) in the substantia nigra. Rotenone reduced intracellular ATP levels in the striatum (by >40%), increases malondialdehyde (MDA, indicative of lipid peroxidation, by ~60%), reduced GSH levels (by ~20%), thioredoxin (by ~70%), and manganese superoxide dismutase (SOD, by ~15%) (all parameters significantly changed in the striatum). Antioxidant polydatin (Piceid) treatment significantly prevented the rotenone-induced changes by restoring the above parameters to control levels, confirming that rotenone- induced mitochondrial dysfunction resulted in oxidative stress (Chen et al., 2015).
- Rotenone was administered 2.5 mg/kg body weight to male Wistar rats for 4 weeks in the presence or absence of ferulic acid (FA, at the dose of 50 mg/kg) that has antioxidant and anti-inflammatory properties. Rotenone administration caused DA neuronal cell death (~50%), significant reduction in endogenous antioxidants, such as superoxide dismutase (~75%), catalase (~40%), and glutathione (~50%), and induced lipid peroxidation evidenced by increased MDA formation (~2 folds). Treatment with FA rescued DA neurons in substantia nigra pars compacta area and nerve terminals in the striatum, as well as restored antioxidant enzymes, prevented depletion of glutathione, and inhibited lipid peroxidation induced by rotenone (Ojha et al., 2015).
- Many studies have shown that mitochondrial aldehyde dehydrogenase 2 (ALDH2) functions as a cellular protector against oxidative stress by detoxification of cytotoxic aldehydes. Dopamine is metabolized by monoamine oxidase to yield 3,4-dihydroxyphenylacetaldehyde (DOPAL) then converts to a less toxic acid product by ALDH. The highly toxic and reactive DOPAL has been hypothesized to contribute to the selective neurodegeneration of DA neurons. In this study, rotenone (100 nM, 24 hr) in both SH-SY5Y cells and primary cultured substantia nigra (SN) DA neurons, was shown to reduce DA cell viability (~40%), reduce Δψm (~40%, as shown by TMRM), induce mitochondrial ROS production (~30%, as shown by increase of MitoSox Red), and increased cytosolic protein levels of proteins related to the mitochondrial apoptotic pathway (i.e. Bax, cytochrome c, active caspase-9 and active caspase-3) (~ 2 folds for all proteins).

The neuroprotective mechanism of ALDH2 was observed as overexpression of wild-type ALDH2 gene (but not the enzymatically deficient mutant ALDH2*2 (E504K)) reduced rotenone-induced DA neuronal cell death, prevented rotenone-induced reduction in TMRM signal (95.7±1.6% v.s. 67±3.5%), and prevented rotenone-induced increase in MitoSox Red intensity (103.1±1% v.s. 133.4±0.8%). Additionally, pre-treatment of cells with Alda-1 (activator of ALDH2) (1–10 μM, for 24 hr) prevented rotenone-induced loss of Δψm and ROS production in a dose-dependent manner. These results were confirmed by in vivo studies. Rotenone (50 mg/kg/day, oral administration for 14 days) or MPTP (40 mg/kg/day, i.p. for 14 days) both administered to C57BL/6 mice caused significant SN TH+ DA neuronal cell apoptosis (~50%). Alda-1 attenuated rotenone-induced apoptosis by decreasing ROS accumulation, reversing Δψm depolarization, and inhibiting the activation of proteins related to mitochondrial apoptotic pathway. The present study demonstrates that rotenone or MPP+ induces DA neurotoxicity through oxidative stress. Moreover, Alda-1 is effective in ameliorating mitochondrial dysfunction by inhibiting rotenone or MPP+ induced mitochondria-mediated oxidative stress that leads to apoptosis (Chiu et al., 2015).

- Rotenone-induced mitochondrial dysfunction was observed in human neuroblastoma cells exposed to 5 nM rotenone for 1-4 weeks. After 3-4 weeks of treatment, rotenone-treated cells showed evidence of oxidative stress, including loss of GSH (by 5%) and increased oxidative DNA (qualitative, measured by using antibodies to 8-oxo-dG) and protein damage (223 ± 29% of control, as shown by the large increase in protein carbonyls in the insoluble fraction) (Sherer et al. 2002). This chronic rotenone treatment markedly sensitized cells to further oxidative challenge since in response to H2O2 cytochrome c release from mitochondria and caspase-3 activation occurred earlier and to a greater extent in rotenone-treated cells vs Ctr (1.44 ± 0.02% vs 0.38 ± 0.07% apoptosis/hr). This study indicates that chronic, low-level CI inhibition by rotenone induces progressive oxidative damage, and caspase-dependent neuronal cell death (Sherer et al., 2002).
- By using anti-oxidant, kaempferol (6 μM, 1 hr prior addition of rotenone) and rotenone (50 nM, max up to 24 hr) on SH-SY5Y cells, kaempferol was found to counteract rotenone-induced ROS production

(especially superoxide: with kaempferol, ethidium fluorescence decreased below the control (Ctr) levels), rotenone-induced mitochondrial oxidative dysfunction (protein carbonyls values: 2.5 in Ctr, 6.2 with rotenone, 2.7 with kaempferol + rotenone), rotenone-induced oxygen respiration (values of nmol of atomic oxygen/minute/mg protein: 5.89 Ctr, 0.45 with rotenone, 2.47 with kaempferol + rotenone), rotenone-induced $\Delta\psi m$ decrease (~70% cells of with rotenone only vs ~30% with kaempferol + rotenone) (Filomeni et al., 2012).

- To model the systemic mitochondrial impairment, rats were exposed to rotenone. A single rotenone dose (10 nM, for 24 hr) induced mtDNA damage in midbrain neurons (>0.4 lesions/10kb vs 0 lesions/10kb in vehicle), but not in cortical neurons; similar results were obtained in vitro in cultured neurons. Importantly, these results indicate that mtDNA damage is detectable prior to any signs of neuronal degeneration and is produced selectively in midbrain neurons. The selective vulnerability of midbrain neurons to mtDNA damage was not due to differential effects of rotenone on CI since rotenone suppressed respiration equally (~60%) in midbrain and cortical neurons compared to vehicle. However, in response to CI inhibition, midbrain neurons produced more mitochondrial H2O2 (5 min of rotenone increased MitoPY1 fluorescence of ~10% in midbrain mitochondria vs vehicle, and progressively for the duration of measurement), than cortical neurons. The selective mtDNA damage in midbrain could serve as a molecular marker of vulnerable nigral neurons in PD. Oxidative damage to cell macromolecules in human PD and the rotenone model have been recently reviewed (Sanders et al., 2014).
- Adult male Sprague–Dawley rats were intranigrally infused with rotenone (6 μg in 1 μl) alone or in the presence of L-deprenyl (0.1, 1, 5 and 10 mg/kg; i.p.) at 12 h intervals for 4 days. Rotenone alone (100 μM, 30 min) increased the levels of hydroxyl radials in the mitochondrial P2 fraction 2,3-DHBA (122.90 ± 5.4 pmol/mg protein) and 2,5-DHBA (146.21 ± 6.3 pmol/mg protein). L-deprenyl (100 nM–1 mM) dose-dependently attenuated rotenone-induced ·OH generation in the mitochondrial P2 fraction. L-deprenyl-induced attenuation in the rotenone-mediated 2,3-DHBA generation was from 17 ± 1.1% to 67 ± 4.3%, respectively, for 100 nM–1 mM of the MAO-B inhibitor. Also, rotenone caused about 51 ± 3.3% reduction in GSH levels in the cell body region, SN and 34 ± 1.1% decrease in the nerve terminal region, NCP (nucleus caudatus putamen). L-deprenyl alone did not cause any significant difference in the GSH content in either region. L-deprenyl treatment dose-dependently attenuated the rotenone-induced GSH depletion in SN from 51 ± 3.1% to 44 ± 2.1%, 32 ± 1.7% and 9 ± 1.0%, respectively, for doses of 1, 5 and 10 mg/kg. Additionally, SOD activity was assayed in rotenone-lesioned animals, which were treated with l-deprenyl at different doses (1–10 mg/kg). SN exhibited 2- and 3-fold activity of Cu/Zn-SOD (i.e. cytosolic SOD fraction) and Mn-SOD (i.e. particulate SOD fraction), respectively, compared to the nerve terminal region, NCP. L-deprenyl (5 and 10 mg/kg) in rotenone-lesioned animals caused a significant increase in the cytosolic Cu/Zn SOD activity in SN of both the sides. Intranigral infusion of rotenone alone caused a significant increase in the enzyme activity in SN of the side of infusion as compared to the non-infused side (~20%). L-deprenyl (5 and 10 mg/kg) further increased catalase activity in both ipsilateral SN and striatum, as compared to the contralateral side of infusion. Finally, rotenone caused a 74% reduction in the striatal TH staining intensity, which was partially recovered by L-deprenyl. These results showed that oxidative stress is one of the major causative factors underlying DA neurodegeneration induced by rotenone and they support the view that L-deprenyl is a potent free radical scavenger and an antioxidant (Saravanan et al., 2006). Similar results were obtained after exposure to MPP+ (Wu et al., 1994).
- Antioxidant (Piperaceae; PLL) with some anti-inflammatory activities demonstrated in preclinical studies protective effects in PD animal models. Rats treated with rotenone and PLL-derived alkaloids showed decreased ROS, stabilized $\Delta\psi m$, and the opening of the mitochondrial PTP - which is triggered by ROS production - was inhibited. In addition, rotenone-induced apoptosis was abrogated in the presence of these alkaloids (Wang H. et al., 2015).
- In SK-N-MC human neuroblastoma cells, rotenone (10 nM - 1μM, 48 hr) caused dose-dependent ATP depletion (~35% reduction by 100 nM rotenone vs Ctr), oxidative damage (100% increase of carbonyls levels upon 100 nM rotenone), and death (100 nM rotenone after 48 hr caused 1.1 AU (arbitrary units) increase of cell death vs untreated – 0.00 AU -). α-Tocopherol pre-treatment (62.5 or 125 μM 24 hr before rotenone (10 nm)) attenuated rotenone toxicity (Sherer et al., 2003).

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or MPP+ (1-methyl-4-phenyl-pyridinium ion)

- MPTP converted into MPP+ inhibits mitochondrial CI activity, resulting in excessive intracellular ROS production followed by further mitochondrial dysfunction leading to mitochondrial-dependent apoptosis. Lutein, a carotenoid of xanthophyll family (antioxidant) reversed MPTP-induced mitochondrial dysfunction, oxidative stress, apoptotic cell death and motor abnormalities. These results revealed that antioxidant protected DA neurons and diminished mitochondrial dysfunction and apoptotic death (Nataraj et al., 2015).
- Antioxidant (salidroside; Sal) pre-treatment protected DA neurons against MPTP/MPP+ induced toxicity in a dose-dependent manner by: (1) reducing the production of ROS, (2) regulating the ratio of Bcl-2/Bax, (3) decreasing cytochrome-c and Smac release, and inhibiting caspase-3, caspase-6, and caspase-9 activation, which are known to trigger apoptosis following mitochondrial dysfunction. Sal acted as an effective neuroprotective agent through modulation of the ROS-induced mitochondrial dysfunction in vitro and in vivo (Wang S. et al., 2015).

- In an in vitro study, MPP+ (1 mM, 24 hr) was found to elicit production of ROS (by 2 fold vs Ctr) and reduce by 50% SOD (by about 50%) and catalase (by about 65%) activity in SH-SY5Y human neuroblastoma cells. Pre-treatment with the antioxidant astaxanthin (AST; 50 µM, 24 hr) inhibited MPP+-induced production of ROS and attenuated both SOD and catalase activity decrease. Furthermore, MPP+ (1 mM, 48 hr) increased caspase-3 activity to 243% of the Ctr and also increased cleaved caspase-3 in the cells (qualitative). Addition of 50 µM AST attenuated MPP+-induced caspase-3 activation (57% suppression). MPP induced also a 70% reduction of Δψm and cytochrome c release (qualitative), while AST prevented both these effects. The protective effects of AST on MPP+ induced mitochondrial dysfunction was due to its anti-oxidative properties and anti-apoptotic activity via induction of expression of SOD and catalase (as shown above) and regulating the expression of Bcl-2 and Bax (Bax/Bcl-2 ratio increased to 1.6-fold vs Ctr upon treatment with MPP+, while AST prevented the MPP+-induced increase of the Bax/Bcl-2 ratio). These results were confirmed by in vivo studies (Lee et al., 2011).
- DA neurons in primary mesencephalic cultures treated with MPP+ (100 µM, for 48 hr) underwent reduction of cell viability (~55% MTT reduction), LDH release (~90%), about 60% reduction of TH+ cells, disruption of Δψm (~45% decline) and ROS production (~60% increase), upregulation of Nox2 (~45%) and Nox4 (~60%), while promoting a decrease of both SOD (~45%) and GSH activity (~85%). Additionally, MPP induced apoptosis via mitochondrial dysfunction, as shown by induction of cytochrome c (~55%), cleaved-caspase-3 (~75%), upregulation of Bax expression (~55%), and downregulation of Bcl2 (~60%). Liuwei dihuang (LWDH), a widely used traditional Chinese medicine (TCM), has antioxidant characteristics. LWDH-WH, derivative of LWDH (0.01-10 µg/ml, added 1 hr prior to MPP+ addition) reduced oxidative damage via increasing antioxidant defence (SOD, GSH), decreasing ROS production, and down-regulating NADPH oxidases (Nox2 and Nox4). LWDH-WH also inhibited neuronal apoptosis by increasing anti-apoptotic protein Bcl-2 expression, and down-regulating apoptotic signalling (Bax, cytochrome c, cleaved-caspase-3) in MPP+-treated neurons. All these protective effects were induced in a dose-dependent manner (Tseng et al., 2014).
- PC12 cells treated with MPP+ (500 µM, for 24 hr) underwent reduction of viability (~55% MTT reduction), oxidative stress (~160% increase in ROS production) and downregulation of heme oxygenase-1 expression (~ 2 folds). Pre-treatment with edaravone, a novel free radical scavenger, (25, 50, 75, 100 µM, for 1 h prior MPP+ treatment) protected PC12 cells against MPP+-cytoxicity via inhibiting oxidative stress and up-regulating heme oxygenase-1 expression in a dose-dependent manner (Cheng et al., 2014).
- The protective effects of antioxidant, apigenin (AP), naturally occurring plant flavonoids were observed on the MPP+ induced cytotoxicity in cultured rat adrenal pheochromocytoma cells (PC12 cells). The PC12 cells were pre-treated with various concentrations of the test compound for 4 h, followed by the challenge with 1,000 µM MPP+ for 48 h. Pre-treatment with AP (3 - 6 - 12 µM) before MPP+ significantly reduced the level of intracellular ROS and elevated Δψm in the MPP+ treated PC12 cells. In addition, AP markedly suppressed the increased rate of apoptosis and the reduced Bcl 2/Bax ratio induced by MPP+ in the PC12 cells. The findings demonstrated that AP exerts neuroprotective effects against MPP+ induced neurotoxicity in PC12 cells, at least in part, through the inhibition of oxidative damage and the suppression of apoptosis through the mitochondrial pathway (Liu et al., 2015).
- Brain mitochondria isolated from ventral midbrain of mitochondrial matrix protein cyclophilin D (CYPD) knockout mice were significantly less sensitive to acute MPP+ (20 µM) -induced effects. CYPD ablation attenuated in vitro Ca2+-induced mitochondrial dysfunction and ROS generation upon Ca2+ loading, both in the absence and in the presence of MPP+, compared to wild-type mice. CYPD ablation conferred a protection to mitochondrial functions upon in vivo treatment with MPTP.

Ventral midbrain mitochondria (that constitutes < 5% of SNpc DA neurons) isolated from brains of wild type (wt) mice acutely treated with MPTP (single MPTP 20 mg/kg injection, analysis done after 4 hr), as compared with saline-treated mice, showed a reduction of CI (by 53%), a reduced rate of phosphorylating respiration (by 38%), a reduced respiratory control index (by 37%), and a decreased ADP/O ratio (by 18%). Ventral midbrain mitochondria isolated from brains of CYPD knockout mice acutely treated with MPTP, as compared with MPTP-treated wt mice, exhibited higher activity of CI (~80%, vs 53% wt), higher rate of phosphorylating respiration (~82%, vs 62% wt), a better respiratory control index (~79%, vs 63% wt), and a higher ADP/O ratio (~90% vs 82% wt) (Thomas et al., 2012). CYP plays as a regulatory component of a calcium-dependent permeability transition pores (PTP), and the data suggest that PTP is involved in MPP+-induced mitochondrial damage. Under oxidative stress, the prolonged opening of the PTP results in calcium overload and with time mitochondrial dysfunction as they get de-energized, depolarized, triggering apoptotic or necrotic cell death (Bernardi, 1999).

There are many other studies showing that MPP+ induces NADH-dependent SOD formation and enhances NADH-dependent lipid peroxidation in submitochondrial particles, confirming that oxidative stress is induced by MPP+ (e.g. Takeshige, 1994; Ramsay and Singer, 1992).

Based on the human post mortem studies of PD brains it is well established that oxidative stress and mitochondrial dysfunction accompany the pathophysiology of PD (e.g. Dias et al., 2013; Zhu and Chu, 2010; Hartman et al., 2004; Fujita et al., 2014).

Examples of human data confirming the presence oxidative stress and mitochondrial dysfunction in PD post mortem brains:

- A significant decrease in CI activity has been identified in a large study of post-mortem PD brains, specifically in substantia nigra compared with age matched controls. In idiopathic PD all 10 patients studied had significant reductions of CI activity (Parker et al., 1989). It is hypothesize that the CI dysfunction may have an etiological role in the pathogenesis of PD (Greenamyre et al., 2001; Sherer et al., 2003, Schapira et al., 1989).
- The structure and function of mitochondrial respiratory-chain enzyme proteins were studied post-mortem in the substantia nigra of nine patients with PD and nine matched controls. Total protein and mitochondrial mass were similar in the two groups. CI and NADH cytochrome c reductase activities were significantly reduced, whereas succinate cytochrome c reductase activity was normal. These results indicated a specific defect of CI activity in the substantia nigra of patients with PD (Schapira et al., 1990).
- Post mortem human studies show that CI deficiency in PD is anatomically specific for the substantia nigra, and they are not present in another neurodegenerative disorder involving the substantia nigra. These results suggest that CI deficiency may be the underlying cause of DA cell death in PD (Schapira et al., 1990; Schapira, 1994).
- The mitochondrial respiratory chain function was studied in various brain regions as well as in skeletal muscle and in blood platelets from patients with idiopathic PD and from matched controls. The evidence suggests that the CI deficiency in PD is limited to the brain and that this defect is specific for the substantia nigra (Mann et al., 1992).
- Immunoblotting studies on mitochondria prepared from the striata of patients who died of PD were performed using specific antisera against Complexes I, III and IV. In 4 out of 5 patients with PD, the 30-, 25- and 24-kDa subunits of CI were moderately to markedly decreased. No clear difference was noted in immunoblotting studies on subunits of Complexes III and IV between the control and PD. The authors claim that deficiencies in CI subunits seem to be one of the most important clues to elucidate pathogenesis of PD (Mizuno et al., 1989).
- Redox markers have been found unchanged in PD patient-derived vs Ctr-derived fibroblasts at baseline. Basal mitochondrial respiration and glycolytic capacity resulted similar at baseline between PD and Ctr fibroblasts, while rotenone-sensitive respiration (analysed by using 0.5 μM rotenone) resulted lower in PD fibroblasts vs Ctr (174.74 ± 48.71 vs 264.68 ± 114.84) (Ambrosi et al., 2014).
- Augmented oxidative metabolism has been detected in PD brains by magnetic resonance studies, in conjunction with energy unbalance. Decreased glucose consumption (22% mean reduction), likely reflecting a decrease in neuronal activity, has been reported in the nigrostriatal system of PD patients (Piert et al., 1996). These symptoms were hypothesized to be indicative of mitochondrial dysfunction as early markers, present in the brain of patients with PD even in the absence of overt clinical manifestations (Rango et al., 2006). In particular, by using high temporal and spatial resolution 31P magnetic resonance spectroscopy (31P MRS) technique authors studied mitochondrial function by observing high-energy phosphates (HEPs) and intracellular pH in the visual cortex of 20 PD patients and 20 normal subjects at rest, during, and after visual activation. In normal subjects, HEPs remained unchanged during activation, but rose significantly (by 16%) during recovery, and pH increased during visual activation with a slow return to rest values. In PD patients, HEPs were within the normal range at rest and did not change during activation, but fell significantly (by 36%) in the recovery period; pH did not reveal a homogeneous pattern with a wide spread of values. Energy unbalance under increased oxidative metabolism requirements, that is, the post-activation phase, discloses a mitochondrial dysfunction that is present in the brain of patients with PD even in the absence of overt clinical manifestations,(Rango et al., 2006).

There are many other studies providing evidence that oxidative stress and mitochondrial dysfunction play an important role in PD pathophysiology (see indirect KER Mitochondrial dysfunction induced DA neuronal cell death of nigrastriatal pathway).

Uncertainties or Inconsistencies

Some studies suggest that rotenone may have effects other than CI inhibition, and it has been claimed that rotenone induces microtubule disruption, rather than ETC CI inhibition (Feng, 2006; Ren et al., 2005). Some studies suggested that there was no evidence for significant change in mitochondrial CI function in PD patients' brains (Jenner et al., 1992). It is still unclear whether the site of superoxide production in CI inhibited mitochondria is CI itself or not (Singer and Ramsay, 1994).

Quantitative Understanding of the Linkage

Based on the available data, the threshold effect seen in brain mitochondria indicates that modest CI inhibition (~ 25-50% decrease in activity) may not directly impact ATP levels or Δψm. Indeed, low levels of CI inhibition produces an oxidative stress without any significant changes in mitochondrial respiration (Betarbet et al., 2000; Greenamyre et al., 2001) or causes not significant changes in ATP levels (Sherer et al., 2003). In particular, in rotenone-infused animals (2.0 mg/kg per day for 2 days), [3H] dihydrorotenone binding to CI in brain was reduced by about 73%. Based on this degree of binding inhibition, the rotenone concentration in brain was estimated to be between 20–30 nM. Complexes II and IV were unchanged by rotenone infusion (Betarbet et al., 2000).

However, such defects have long-term deleterious effects. It is well documented that that there is a site of electron leak upstream of the rotenone binding site in CI (i.e., on the ‘NADH side’ of the complex) (Hensley et al., 1998) leading to the superoxide (O2-) and followed up by H2O2 production by CI (Greenamyre et al., 2001). The relative role of each ETC complex in forming superoxide differs by tissue; however CI is a major source of O2- in the brain (Halliwell, 2007).

Thus, a low inhibition of CI activity that is insufficient to affect cell respiration may lead to mitochondrial damage and chronic up-regulation of ROS production. Therefore, it is suggested that rotenone that binds to CI with an affinity of 10-20 nM induces toxicity not by bioenergetics effects but rather via accumulative oxidative stress. Sustained oxidative stress leads to decrease levels of reduced glutathione; activation of superoxide dismutase (SOD) (scavenger of O2-), catalase and indeed, treatments with antioxidants reduce the oxidative stress-induced damage. Such data are abounded in the existing literature based both on in vivo and in vitro studies and a few examples are described in the Empirical support for linkage.

The selective CI defects (other complexes were unaffected) (Schapira et al., 1990a) and induced mitochondrial damage followed by oxidative stress is also described in PD patients brains as documented by: (a) reduced glutathione levels (Jenner at al., 1992); (b) increased content of 8-oxo-deoxyguanine, a marker of oxidatively damaged nucleic acids (Alam et al., 1997; Mecocci et al., 1993); (c) increased level of malondialdehyde (marker of lipid peroxidation) (Navarro et al., 2009); (d) increased cholesterol lipid hydroperoxide (Dexter et al., 1994); (e) increased protein oxidation measured e.g. by elevated levels of methionine sulfoxide formation or protein carbonyl content (Alam et al., 1997). These studies in human brain present a semiquantitataive evaluation of the oxidative stress, as there is no data showing KER between the various degrees of CI inhibition and mitochondrial damage (ROS production) and the parameters described above. However, these studies clearly confirmed that oxidative stress in PD patient brain is increased as shown by the measured biomarkers (Sanders and Greenamyre, 2013).

In in vitro and in in vivo animal studies there are some data showing the quantitative relationship between the oxidative stress produced by inhibition of CI and mitochondrial damage measured by the same assays, as described in human studies, and a few examples of such experiments are discussed below. The quantitative evaluation of the causative relationship between the CI inhibition (KE up) induced by rotenone (4 hr exposure) and mitochondrial dysfunction (KE down) measured in human-chimpanzee isolated mitochondria (xenomitochondrial cybrids; HXC) by a decreased cell respiration and Δψm, increased ROS production and lipid peroxidation showed linear, time- and concentration-dependent effects (below Fig.1 from Barrientos and Moraes, 1999).

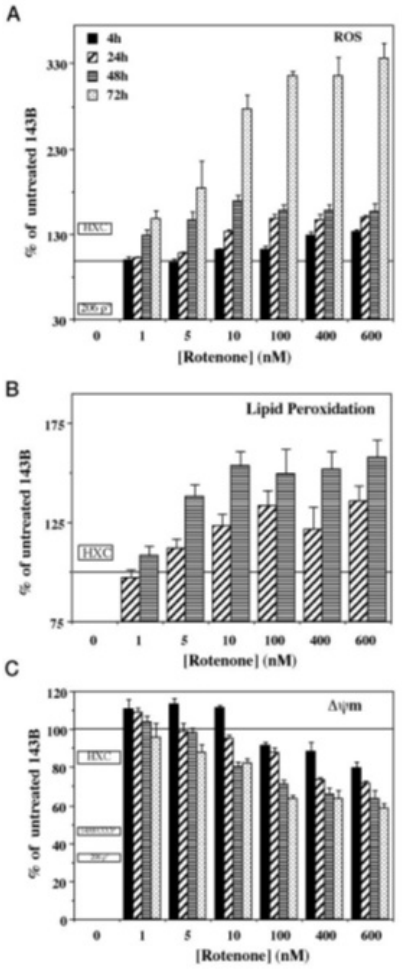

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Fig.1. A dose- and time-dependent effect of CI inhibition by rotenone on (A) reactive oxygen species production (ROS), (B) Lipid peroxidation and (C) mitochondrial membrane potential (Δψm) studied in the human

osteosarcoma-derived cell line (143B) or using a genetic model (40% CI inhibited in HXC lines) (for further information see Barrientos and Moraes, 1999).

- The endogenous respiration was inhibited in a dose-dependent manner but showed different inhibition kinetics. Only when CI was inhibited by 35-40% (< 5 nM rotenone), cell respiration started decreasing (a threshold for inhibition for cell respiration triggered by rotenone). Between 40 and 60% of CI inhibition (5-10 nM), cell respiration decreased linearly until 30% of the normal rate. Increasing concentrations of rotenone produced further but slower decrease in CI activity and cell respiration. 100% CI inhibition was achieved with 100 nM rotenone but the cells still maintained a cell respiration rate (through complex II), approximately 20% and the rate of ROS production increased by a maximum of 20-25% (4 hr treatment). ROS production was saturated at 100 nM rotenone but an initial effect was observed already at 1-5 nM (Barrientos and Moraes, 1999). Inhibition of CI activity triggered decrease of cell respiration by different concentrations of rotenone and resulted in mitochondrial damage measured not only by ROS production, but also by lipid peroxidation and decreased Δψm. Inhibition of CI by 25, 50, 75 and 100 % decreased cell respiration by 5, 20, 53, 81 %, increased ROS production by 48, 81, 157, 216%, increased lipid peroxidation by 8, 27, 45, 55 % and decreased Δψm by 6, 13, 20, and 37% respectively (approximately).

Similar studies were also performed using different types of neuronal cells.

- Hoglinger and colleagues, by using DA neurones derived from the rat (embryonic day 15.5) ventral mesencephalon, showed that CI inhibition by rotenone at 30 nM, (or MPP+ 3 µM) for 24 hr decreased ATP levels (by > 80%) within the first 6 hr, and neuronal cell death within 24 hr. When residual ATP levels remained above 20%, there was no or little neuronal loss, suggesting that 20% of normal ATP level was the minimum compatible with neuronal survival. Rotenone (and MPP+) increased ROS (≥40% over control levels) already at low concentrations that were subtoxic or only moderately toxic (i.e., 10-30 nM for rotenone, 10-30 µM for MPP+) (Fig. 2) (Hoglinger et al., 2003).

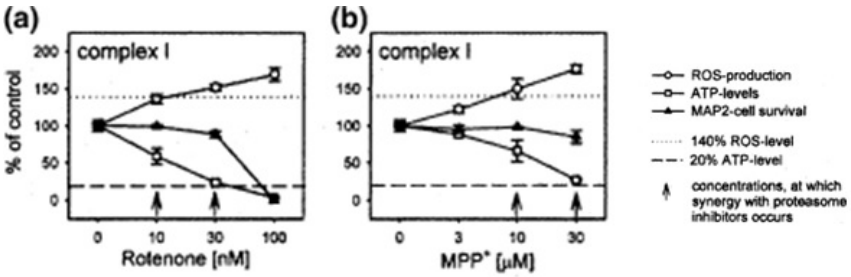

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Fig. 2. ATP levels, ROS production and neuronal surviving cells in mesencephalic cultures treated with CI inhibitors (rotenone and MPP+) (from Hoglinger et al., 2003, Fig. 4a-b)

- Shamoto-Nagai and colleagues showed that 25 or 50 nM rotenone decreased ATP levels over time. In particular, the intracellular ATP level was reduced to 18.0% and 19.6% of control after 44 hr of treatment with 25 and 50 nM of rotenone, respectively, and thereafter the decreased level was sustained (Fig. 3, left) (Shamoto-Nagai et al., 2003). Also, The production of ROS-RNS increased 6 hr after the rotenone treatment, and the increase was about 1.5-fold of the basal value. With treatment with the higher (50 nM) concentration of rotenone, DCF production level was restored to the basal level after 48 hr, whereas, at the lower concentration (25 nM), DCF production increased again at 48 hr and then declined to the basal value after 90 hr (Fig. 3, right) (Shamoto-Nagai et al., 2003).

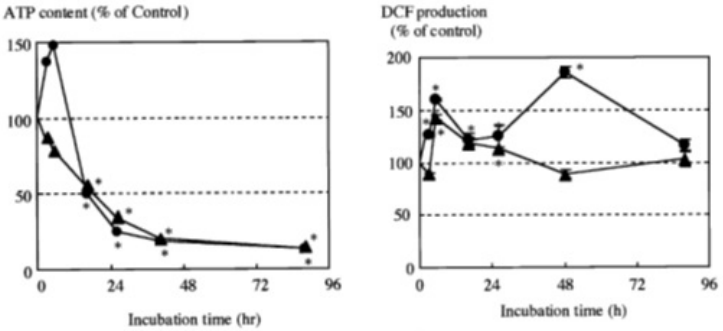

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Fig. 3. Effect of rotenone on ATP level (left) and on ROS and RNS production (right) in SH-SY5Y cells. SH-SY5Y cells were treated with 25 nM (circles) or 50 nM (triangles) of rotenone. * indicates significant difference from control (P < .05) (from Shamoto-Nagai et al., 2003, Figs. 2, 3)

- Human neuroblastoma cell line (SK-N-MC) exposed to 5 nM rotenone chronically, for 4 weeks caused reduction in GSH by 44%, GSSG by 40%. These effects were not observed after two weeks of exposure. Total cellular GSH levels were reduced after 4 weeks of exposure by 50% (Sherer et al., 2002). Similarly, in the same study, 1-2 weeks of treatment did not alter protein carbonyl levels (oxidative protein damage) but exposure for 3-4 weeks caused a large increase in carbonyls in the insoluble fraction by approximately 223% of control. Systemic in vivo rotenone infusion (up to 5 weeks, 3.0 mg/kg/day) modestly elevated soluble protein carbonyls in the rat cortex by approximately 19%, in the striatum by 27% and the largest elevation occurred in the DA neurons of midbrain, around 41% (no effect in cerebellum or hippocampus) (Sherer et al., 2003).

The prolonged treatment with rotenone (3-4 weeks, not 1-2 weeks) caused also a marked increase in 8-oxo-dG immune-reactivity (i.e., oxidative DNA damage) and redistribution of cytochrome c (Sherer et al., 2002).

- The same group showed that exposure of SK-N-MC cells for 6-8 hr to low concentrations of rotenone (100 pM, 1 nM, 10 nM and 100 nM) produced a concentration-dependent decrease in ATP levels by 0, 2.5, 10, and 32.2 % respectively (Sherer et al., 2003).
- The oxidative stress (mitochondrial damage) induced by rotenone exposure was confirmed in ex-vivo studies using brain sections at the level of the substantia nigra that were treated with 50 nM rotenone over 1 week. A significant increase of protein carbonyls (indicative of oxidative damage to proteins; biomarkers of oxidative stress) was observed (~ 25%) when compared to the untreated slices. Exposure to 100 µM α-tocopherol, antioxidant (vitamin E) significantly protected the neurons from the oxidative damage induced by 50 nM rotenone over 1 week (~ 25%), as shown by lower protein carbonyl levels (~ 3%), with very similar effects observed with 20 nM rotenone over 2 weeks (Testa et al., 2005).

The same assays for mitochondrial dysfunction evaluation after exposure to rotenone, MPTP or other chemicals were used through a range of different studies (Sherer et al., 2003, Betarbet et al., 2000) and the role of CI inhibition in PD is discussed in many published reviews (Sanders and Greenamyre, 2013, Greenamyre et al., 2001, Schapira et al., 1990a and 1990b).

Summing up, it is well documented in human PD brain studies as well as in vivo and in vitro existing data that CI inhibition induces mitochondrial dysfunction as shown by measuring the decreased cellular respiration and induced oxidative damage to protein, lipids and nucleic acids, as well as compromised function of anitioxidant defense mechanisms (e.g. decreased levels of reduced glutathione). As discussed above, oxidative damage is largely reversed by antioxidants treatments. These data are largely semi quantitative only, as the full dose- and time response curves are available. They indicate that low levels of CI inhibition for long periods of time (4-5 weeks) mostly increase ROS production, having negative effects on DA neurons in SNpc, which seem to be affected more than other neuronal cell types in other brain structures (reviews e.g. by Sanders and Greenamyre, 2013; Greenamyre et al., 2001, Schapira et al., 1990a and 1990b etc.).

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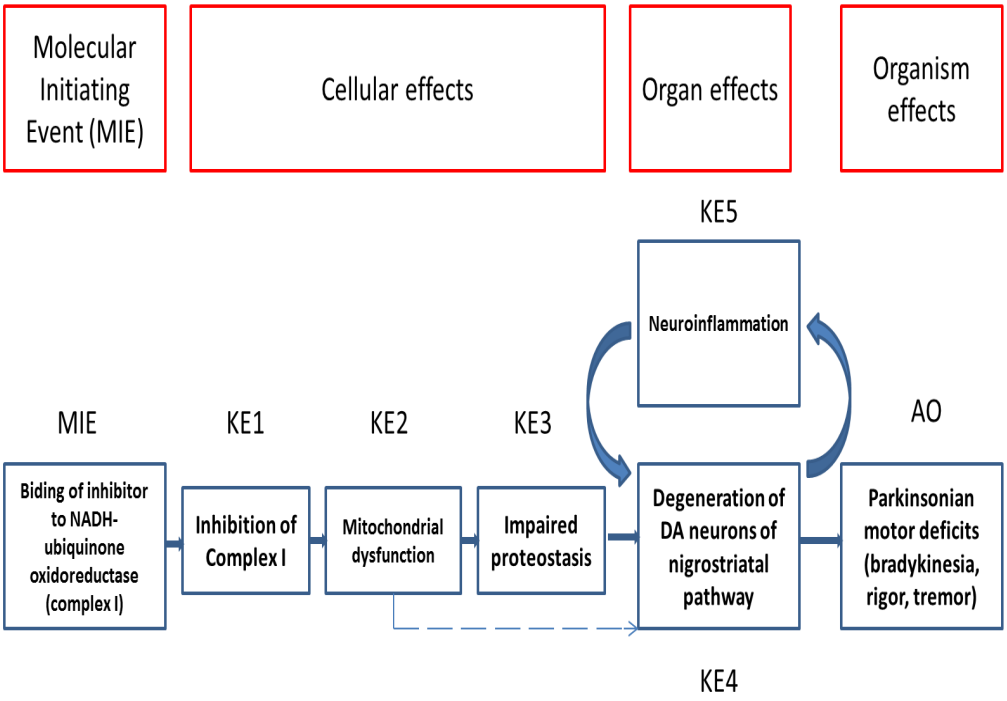
Graphical Representation



Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Mixed	Strong

This proposed AOP is neither sex-dependent nor associated with certain life stage; however, aged animals may be more sensitive. The relevance of this AOP during the developmental period has not been investigated. In vivo testing has no species restriction. The mouse was the species most commonly used in the experimental models conducted with the chemical stressors; though experimental studies using alternative species have been also performed. (Johnson et al. 2015). However, animal models (rodents in particular) would have limitations as they are poorly representative of the long human life-time as well as of the human long-time exposure to the potential toxicants. Human cell-based models would likely have better predictivity for humans than animal cell models. In this case, toxicokinetics information from in-vivo studies would be essential to test the respective concentrations in-vitro on human cells.

Essentiality of the Key Events

Essentiality of KEs for this AOP is strong. There is ample evidence from knock out animal models, engineered cells or replacement therapies that blocking, preventing or attenuating an upstream KE is mitigating the AO. In addition, there is experimental support for the KERs as multiple studies performed with modulating factors that attenuate (particularly with antioxidants) or augment (e.g. overexpression of viral-mutated α-synuclein) a KE show that such interference leads to an increase of KE down or the AO.

2 Support for Essentiality of KEs	**Defining Question** Are downstream KEs and/or the AO prevented if an upstream KE is blocked ?	**High (Strong)**	**Moderate**	**Low(Weak)**
		Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs (e.g. stop/reversibility studies, antagonism, knock out models, etc.)	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE leading to increase in KE down or AO	No or contradictory experimental evidence of the essentiality of any of the KEs
KE1 Inhibition of complex I	STRONG	Rationale: Inactivation of the Ndufs 4 gene (knockout mice) that produces CI deficiency causes encephalomyopathy, including ataxia and loss of motor skills (Kruse et al., 2008). NDI1-transducted SK-N-MC cells expressing the rotenone-insensitive single subunit NADH dehydrogenase of yeast (NDI1) that acts as a replacement for the entire CI in mammalian cells were completely resistant to 100 nM rotenone-mediated cell death (at 48 hrs of exposure) indicating that rotenone – induced toxicity requires rotenone biding of CI (Sherer et al., 2003). In all rotenone models, mitochondria CI is inhibited at the dose that cause neurodegeneration (Betarbet et al 2000 and 2006).		

KE2 Mitochondrial dysfunction	STRONG	Rationale: Many studies showing that antioxidants protect the cells against rotenone or MPTP induced oxidative stress are published (Chen et al. 2015; Lu et al., 2015; Saravanan et al., 2006; Chiu et al., 2015, Sherer et al.2003, Nataraj et al.2015, Wu et al. 1994; Tseng et al. 2014; Li et al. 2010; Kim-Han et al. 2011). This provides (indirect) evidence for essentiality of KE2, if production of ROS is assumed as direct consequence/sign of mitochondrial dysfunction. Additional evidence comes from experiments with overexpression or activation of antioxidative enzymes (e.g.SOD or ALDH2) , which also prevent rotenone and MPTP induced neurotoxicity (Mudo et al. 2012; Ciu CC et al. 2015). Furthermore, promotion of mitochondrial fusion or blocking of mitochondrial fission prevents or attenuates rotenone and MPTP induced neurotoxicity (Tieu K. et al. 2014).
KE3 Impaired proteostasis	MODERATE	Rationale: Indirect evidence for the role of disturbed alpha-synuclein proteostasis: Lacking of alpha-synuclein expression in mice prevented induction of behavioural symptoms, neuronal degeneration in the nigrostriatal pathway and loss of DA neurons after chronic treatment with MPTP (Fornai et al. 2004; Dauer et al. 2002) . Injection of adeno/lenti-associated virus that expresses wild-type or mutant α-syn into rat, mice or non-human primate SN produced loss of dopaminergic neurons, but the effect is not easily reproduced in transgenic mice overexpressing alpha-synuclein (Kirk, 2002; Klein, 2002; Lo Bianco, 2002; Lauwers, 2003; Kirk, 2003). Rationale for the role of autophagy: Early dendritic and axonal dystrophy, reduction of striatal dopamine content, and the formation of somatic and dendritic ubiquitinated inclusions in DA neurons were prevented by ablation of Atg7 (an essential autophagy gene (Friedman et al. 2012)). Rationale for the role of UPS/ALP: Protection from DA neuronal death was also observed in multiple experiments through the pharmacological modulation of the UPS, ALP system; however, there are also contradicting data in the literature. (Inden et al. 2007; Fornai et al. 2003; Dehay et al. 2010; Zhu et al. 2007, Fornai et al. 2005). However, although many lines of evidence exist to support essentiality of impaired proteostasis, a single molecular chain of events cannot be established.
KE4 Degeneration of DA neurons of nigrostriatal pathway	MODERATE	Dopaminergic cell death is mostly measured by a decrease in TH expression. However, as a recovery is possible, this may not be associated to irreversible degeneration and is a signal sufficient to trigger microglial reactivity (Sandström et al., 2014) .
KE5 Neuroinflammation	MODERATE	Rationale: Following treatment with Rotenone or MPP+, protection of DA neurons and terminals was observed in vivo and in vitro by inhibiting different feature of neuroinflammation (microglia/astrocyte); however, inhibition was different in different models and considered as an indirect evidence of essentiality (Zhou et al., 2007; Gao et al., 2002 and 2003 and 2015; ; Emmrich et al., 2013; Salama et al., 2012; Chang et al., 2013; Wang et al., 2014; Liu et al., 2012, 2015; Borrajo et al., 2013; Brzozowski et al., 2015; Wang et al., 2006; Chung et al., 2011; Sriram et al., 2014; Feng et al., 2002; Sathe et al., 2012; Khan et al., 2014; Ros-Bernal et al., 2011; Ferger et al., 2004; Chao et al., 2009; Rojo et al., 2010; Qian et al., 2011; Dehmer et al., 2000; Bodea et al., 2014). Mice lacking the type-1 Interferons receptor showed an attenuated pro-inflammatory response and reduced loss of dopaminergic neurons induced by MPTP. The neuro-protective potential was also confirmed by treatment with a blocking monoclonal antibody against type-1A IFN receptor that increased survival of dopaminergic neurons of TH+ (Main et al., 2016).

KE 6 Degeneration of DA neurons of nigrostriatal pathway	STRONG	Rationale: Clinical and experimental evidences show that the pharmacological replacement of the DA neurofunction by allografting fetal ventral mesencephalic tissues is successfully replacing degenerated DA neurons resulting in the total reversibility of motor deficit in animal model and partial effect is observed in human patient for PD (Widner et al., 1992; Henderson et al., 1991; Lopez-Lozano et al., 1991; Freed et al., 1990; Peschanski et al., 1994; Spencer et al., 1992). Also, administration of L-DOPA or DA agonists results in an improvement of motor deficits (Calne et al 1970; Fornai et al. 2005). The success of these therapies in man as well as in experimental animal models clearly confirms the causal role of dopamine depletion for PD motor symptoms (Connolly et al., 2014; Lang et al., 1998Silva et al., 1997; Cotzias et al., 1969; Uitti et al., 1996; Ferrari-Tonielli et al., 2008; Kelly et al., 1987; Walter et al., 2004; Narabayashi et al., 1984; Matsumoto et al., 1976; De Bie et al., 1999; Uitti et al., 1997; Scott et al., 1998; Moldovan et al., 2015; Deuschl et al., 2006; Fasano et al., 2010; Castrito et al., 2011; Liu et al., 2014; Widner et al., 1992; Henderson et al., 1991; Lopez-Lozano et al., 1991; Freed et al., 1990; Peschanski et al., 1994;Spencer et al., 1992). Furthermore, experimental evidence from animal models of PD and from in-vitro systems indicate that prevention of apoptosis through ablation of BCL-2 family genes prevents or attenuates neurodegeneration of DA neurons (Offen D et al., 1998; Dietz GPH et al. 2002).

Weight of Evidence Summary

Concordance of dose-response relationship

Data from experiments with the stressor compounds rotenone and MPTP (known inhibitors of the mitochondrial Complex I (CI)) reveal a good concordance of the dose-response relationships between the MIE and AO and within KEs. Although the different KEs have been measured using different methodologies, comparison of data from multiple in-vitro/in-vivo studies shows a general agreement in dose-relationship (see table 1 and 2). There is a good consistency when comparing data on KE4 and the AO after exposure to rotenone and MPTP. However, in vivo rodent studies proved that only exposure to low concentrations of rotenone (rat brain concentration between 20-30 nM of rotenone; Betrabet et al., 2000) or MPTP (mice striatum concentration of approximately 12-47 µM MPP+; Fornai et al., 2005; Thomas et al. 2012) after chronic exposure (approximately 5 weeks) reproduced the anatomical, neurochemical behavioural and neuropathological features similar to the ones observed in Parkinson's disease (PD). Because of the variability of experimental protocols used, a clear no-effect threshold could not be established; nevertheless, these brain concentrations of rotenone (20-30 nM) and MPP+ (approximately 12-47µM) could serve as probabilistic thresholds for chronic exposure that could reproduce features of PD as both concentrations trigger approximately a 50% inhibition of Complex I (see table 3). Generally, a strong response-response relationship is observed within studies. Some exceptions for this rule are observed between KE3/KE5 and KE4, likely because of the all biological complexity associated with these KEs. In this AOP, neuroinflammation was considered to have a direct effect on degeneration of DA neurons. However, it was not clear at which conditions it would become a modulatory factor and for practical reasons was not included in table 1, 2 and 3 but considered in the weight of evidence analysis.

Table1 Dose-response and temporality table for rotenone

Concentration	KE1aaa Inhibition of C I	KE2aaa Mitochondrial dysfunction	KE3aaa Impaired proteostasis	KE4 Degeneration of DA neurons of nigrostriatal pathway	AO Parkinsonian motor symptoms
5-10 nM *in-vitro* [1]	+ 4-72 hours [1]	+ 4-72 hours [4]	+ 24 hours [3]	-	-
20-30 nM *ex-vivo*, rat brain concentration [4-5-2-6]	++ 4-72 hours (4-5)	++ 4-72 hours [4-5]	++ 24 hours [3-2-6]	++a 5 weeks [2-6]	+++aa 5 weeks [2-6]

100 nM *in-vitro* [4]	+++ 4-72 hours [4]	+++ 4-72 hours [4]	+++ 24 hours [3]	Above the maximum tolerated dose [2-6]	Above the maximum tolerated dose [2-6]

References: Choi et al. 2008 [1]; Betarbet et al. 2006 [2]; Chou et al. 2010 [3]; Barrientos and Moraes 1999 [4]; Okun et al. 1999 [5]; Betarbet et al. 2000 [6]

-no data available

+: low severity score, ++ intermediate severity score, +++ high severity score

a: 50% of treated animals showed loss of DA neurons in SNpc

aa: All animals affected in KE4 showed impaired motor symptoms

aaa: KE 1, 2 and 3 showed a dose-related severity in the effect and the score ++ was normalized vs. the KE4

Table 2. Dose-Response and Temporality table for MPTP

Dose	Brain Concentration	KE1bb Inhibition of C I	KE2bb Mitochondrial dysfunction	KE3b Impaired proteostasis	KE4 Degeneration of DA neurons of nigrostriatal pathway	AO Parkinsonian motor symptoms
1 mg/kg infusion [1]	-	-	-	+ 4 weeks[1]	+aaa 4 weeks [1]	No effect
5 mg/kg infusion [1]	-	-	-	++ 4 weeks[1]	++aa 4 weeks [1]	+++ 4 weeks [1]
20-30 mg/kg infusion [2, 1]	47µM [2]^ 12µM [1]	+++ 4 hrs [2]	+++ 4hrs [2]	+++ 4 weeks [1]	+++a 1-4 weeks[2,1]	+++ 4 weeks [1]

References. Fornai et al. 2005 [1]; Thomas et al. 2012 [2]

-no data available

a: approx 50% loss of DA neurons in SNpc

aa: approx 30% loss of DA neurons SN pc

aaa: no loss of DA neurons in SN pc. Reduced level of striata DA

b: for KE3, a dose response effect was observed.

bb: for KE 1 and 2 the severity of the effect was normalized vs. the KE4

^ After single dose MPTP administration, brain concentration was approx. 5.15 µM

Temporal concordance among the MIE, KEs and AO

There is a strong agreement that loss of DA neurons of the SNpc that project into the putamen is preceded by reduction in DA and degeneration of DA neuronal terminals in the striatum (Bernheimer et al. 1973). The clinical symptoms of a motor deficit are observed when 80% of striatal DA is depleted (Koller et al. 1992) and the sequence of pathological events leading to the adverse outcome has been well-documented (Fujita, et al.2014; O'Malley 2010, Dexter et al. 2013). Temporal concordance (see table 1 and 2) among the KEs can be observed in the experimental models of PD using the chemical stressors rotenone and MPTP (Betarbet 2000 and 2006; Sherer et al. 2003, Fornai et al. 2005). The acute administration of the chemical stressors can trigger a dose-related change from the MIE to impaired proteostasis; however, to trigger KE4 (i.e. degeneration of DA neurons in SNpc with presence of intracytoplasmatic Lewy-like bodies) and motor deficits (AO), proteostasis needs to be disturbed for a minimum period of time (Fornai et al. 2005).

Strength, consistency, and specificity of association of AO and MIE

Strength and consistency of the association of the AO with the MIE is strong. There is a large body of evidence from in-vitro and in-vivo studies with chemical stressors, showing association between the MIE that triggers an inhibition of CI and the AO (Sherer et al. 2003; Betarbet et al. 2000 and 2006, Fornai et al. 2005). Human data also suggest a link between inhibition of CI and AO (Greenamyre et al. 2001; Schapira et al. 1989; Shults, 2004). Using the two different chemical stressors, rotenone and MPTP, data are consistent and the pattern of activation of the MIE leading of the AO is similar. For rotenone and MPTP, specificity is high; however, there are many inhibitors of the mitochondrial CI without evidence of triggering the AO. When considering these chemicals specificity is low; therefore, kinetic and metabolic considerations should be taken into account to fully demonstrate specificity for these compounds.

Weight of Evidence (WoE)

Biological plausibility, coherence, and consistency of the experimental evidence

The biological plausibility of this AOP is overall considered strong. When using multiple stressors in different studies and assays, the coherence and consistency of the experimental data is well established. Furthermore, in-vivo and in-vitro studies are also in line with the human evidence from PD patients. In addition, although the mechanistic understanding of parkinsonian disorders (and PD in particular) are not fully clear, the KEs and KERs described in this AOP are considered critical for the development of the disease (Fujita et al. 2015, Shulman et al. 2011, Dexter et al. 2013, Dauer et al. 2003).

1 Support for Biological Plausibility of KERs	**Defining Question**	**High (Strong)**	**Moderate**	**Low(Weak)**
	Is there a mechanistic (i.e. structural or functional) relationship between KEup and KE down consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance	The KER is plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established	There is empirical support for a statistical association between KEs but the structural or functional relationship between them is not understood
MIE => KE1 Binding of inhibitor to NADH-ubiquinone oxidoreductase leads of complex I	STRONG	Rationale: As describe in this KER there is an extensive understanding of the functional relationship between binding of an inhibitor to NADH-ubiquinone oxidoreductase (CI) and its inhibition. Different complex I ligands, both naturally occurring, like rotenone (from Derris scandens), piericidin A (from Streptomyces mobaraensis), acetogenins (from various Annonaceae species) and their derivatives, and synthetically manufactured like pyridaben and various piperazin derivatives inhibit the catalytic activity of complex I (Degli Esposti, 1994: Ichimaru et al. 2008; Barrientos and Moraes, 1999; Betarbet et al., 2000).		
KE1 => KE2 Inhibition of complex I leads to mitochondrial dysfunction	STRONG	Rationale: There is extensive understanding of the mechanisms explaining how the inhibition of complex I lead to mitochondrial dysfunction (i.e. failure to produce ATP, increase in production of ROS etc). It is well documented that CI inhibition is one of the main sites at which electron leakage to oxygen occurs resulting in oxidative stress (Efremov and Sazanow, 2011; lauren et al. 2010; Greenamyre et al. 2001). These pathological mechanisms are well studied as they are used as readouts for evaluation of mitochondrial dysfunction (Graier et al., 2007; Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013		
KE2 => KE3 Mitochondrial dysfunction results in impaired proteostasis	MODERATE	Rationale: The weight of evidence supporting the biological plausibility behind the relationship between mitochondrial dysfunction and impaired proteostasis, including the impaired function of UPS and ALP that results in decreased protein degradation and increase protein aggregation is well documented but not fully understood. It is well established that the two main mechanisms that normally remove abnormal proteins (UPS and ALP) rely on physiological mitochondrial function. The role of oxidative stress, due to mitochondrial dysfunction, burdens the proteostasis with oxidized proteins and impairs the chaperone and the degradation systems. This leads to a vicious circle of oxidative stress inducing further mitochondrial impairment (Powers et al., 2009; Zaltieri et al., 2015; McNaught and Jenner, 2001). Therefore, the interaction of mitochondrial dysfunction and UPS /ALP deregulation plays a pivotal role in the pathogenesis of PD (Dagda et al., 2013; Pan et al., 2008; Fornai et al., 2005; Sherer et al., 2002).		

KE2 => KE4 Mitochondrial dysfunction leads to the degeneration of dopaminergic neurons of the nigrostriatal pathway	STRONG	Rationale: Mitochondrial are essential for ATP production, ROS management, calcium homeostasis and control of apoptosis. Mitochondrial homeostasis by mitophagy is also an essential process for cellular maintenance (Fujita et al. 2014). Because of their anatomical and physiological characteristics, SNpc DA neurons are considered more vulnerable than other neuronal populations (Sulzer et al. 2013; Surmeier et al.2010). Mechanistic evidence of mutated proteins relate the mitochondrial dysfunction in familial PD with reduced calcium capacity, increased ROS production, increase in mitochondrial membrane permeabilization and increase in cell vulnerability (Koopman et al. 2012; Gandhi et al. 2009). Human studies indicate mitochondrial dysfunction in human idiopathic PD cases in the substantia nigra (Keeney et al., 2006; Parker et al., 1989, 2008; Swerdlow et al., 1996). In addition, systemic application of mitochondrial neurotoxicants such as rotenone or MPTP leads to a preferential loss of nigrostriatal DA neurons (Langston et al., 1983).
KE3 => KE4 Impaired proteostasis leads to degeneration of DA neurons of the nigrostriatal pathway	MODERATE	Rationale: It is well known that impaired proteostasis refers to misfolded and aggregated proteins including alfa-synuclein, deregulated axonal transport of mitochondria and impaired trafficking of cellular organelles. Evidences are linked to PD and experimental PD models as well as from genetic studies (McNaught et al. 2001, 2003; Tieu et al. 2014; Arnold 2011; Rappold et al. 2014). Strong evidence for degeneration of the nigrostriatal pathway comes from the experimental manipulations that directly induce the same disturbances of proteostasis as observed in PD patients (e.g. viral mutated alpha-synuclein expression) or in chronic rotenone/MPTP models trigger degeneration of the nigrostriatal pathway (Kirk et al. 2003; Betarbet et al. 2000 and 2006; Fornai et al. 2005). However, a clear mechanistic proof for the understanding of the exact event triggering cell death is lacking. There is only moderate evidence showing that interventions that correct disturbances of proteostasis after exposure to rotenone would prevent neuronal degeneration and that the disturbances of proteostasis correlate quantitatively under many conditions with the extent of nigrostriatal neuronal degeneration.
KE4 => KE5 Degeneration of DA neurons of the nigrostriatal pathway leads to neuroinflammation	MODERATE	Rationale: The fact that neuronal injury/death can trigger neuroinflammation is supported by evidence in human and experimental models. The evidence that neuroinflammation triggered by neuronal damage can cause neuronal death (vicious circle), is mostly indirect (blockade of any feature of neuroinflammation) or by analogy (Hirsch and Hunot, 2009; Tansey and Goldberg, 2009; Griffin et al., 1998; McGeer and Mc Geer, 1998; Blasko et al., 2004; Cacquevel et al., 2004; Rubio-Perez and Morillas-Ruiz, 2012; Thundyil and Lim, 2014; Barbeito et al., 2010).
KE5 => KE6 Neuroinflammation leads to degeneration of DA neurons of the nigrostriatal pathway	MODERATE	Rationale: The fact that reactive glial cells (microglia and astrocytes) may kill neurons is well accepted. The mechanisms underlying this effect may include the release of cytotoxic signals (e.g. cytokines) or production of ROS and RNS (Chao et al., 1995 ; Brown and Bal-Price, 2003 ; Kraft and Harry, 2011 ; Taetzsch and Block, 2013). However, the responsible mediators differ from model to model.
KE6 => AO Degeneration of DA neurons of the nigrostriatal pathway leads to parkinsonian motor symptoms	STRONG	Rationale: The mechanistic understanding of the regulatory role of striatal DA in the extrapyramidal motor control system is well established. The loss of DA in the striatum is characteristic of all aetiologies of PD and is not observed in other neurodegenerative diseases (Bernheimer et al. 1973; Reynolds et al. 1986). Characteristic motor symptoms such as bradykinesia, tremor, or rigidity are manifested when more than 80 % of striatal DA is depleted as a consequence of SNpc DA neuronal degeneration (Koller et al. 1992).

Empirical support

Empirical support is strong. Many studies show evidence for the KERs by showing temporal concordance and dose concordance when using different stressors.

3 Empirical support for KERs	**Defining Question** Does the empirical evidence support that a change in the KEup leads to an appropriate change in the KE down? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup higher than that for KE down? Are inconsistencies in empirical support cross taxa, species and stressors that don't align with expected pattern of hypothesized AOP?	**High (Strong)**	**Moderate**	**Low(Weak)**
		Multiple studies showing dependent change in both exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor (ie endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP
MIE => KE1 Binding of inhibitor to NADH-ubiquinone oxidoreductase leads to partial or total inhibition of complex I	STRONG	Rationale: The inhibition of complex I is well documented in a variety of studies using isolated mitochondria or cells as well as in in vivo experiments and in human post mortem PD brains. In many experiments using different inhibitors ie rotenone and MPTP, the observed relationship between the two events was temporal, response and dose concordant (Betarbet et al., 2000 and 2006, Okun et al., 1999, Koopman et al., 2007, Choi et al., 2008, Grivennikova et al., 1997, Barrientos and Moraes 1999).		
KE1 => KE2 Inhibition of complex I leads to mitochondrial dysfunction	STRONG	Rationale: There is a large amount of studies showing that the inhibition of CI inhibition results in mitochondrial dysfunctions in a response and dose dependent manner (Barriento and Moraes, 1999).		
KE2 => KE3 Mitochondrial dysfunction results in impaired proteostasis	STRONG	Rationale: Based on the existing in vitro and in vivo data it is suggested that mitochondrial dysfunction impairs protein homeostasis (impairment of the UPS and ALP system) through oxidative and nitrosative stress resulting in accumulation of misfolded proteins (including α-synuclein), disruption of microtubule assembly and damaged intracellular transport of proteins and cell organelles. A number of studies performed with chemical stressors showed evidence of temporal, response and dose concordance (Chou et al. 2010; Betarbet et al. 2000 and 2006; Fornai et al. 2005).		

KE2 => KE4 Mitochondrial dysfunction directly leads to degeneration of DA neurons of nigrostriatal pathway	STRONG	Rationale: Multiple *in vitro* studies indicate dose and response-response concordance. As most of the studies were conducted *in vitro*, the temporal concordance is difficult to establish; however, can be expected based on the well know temporal sequence of the two KEs. (Park et al., 2014; Choi et al., 2014; Marella et al., 2008; Du et al. 2010; Hajieva et al., 2009; Sherer et al., 2003; Sherer et al., 2007; Wen et al. 2011; Swedlow et al., 1996; Jana et al., 2011; Jha et al., 2000; Chinta et al., 2006)
KE3 => KE4 Impaired proteostasis leads to degeneration of DA neurons of the nigrostriatal pathway	STRONG	Rationale: The empirical support linking impaired proteostasis with degeneration of DA neurons of the nigrostriatal pathway is strong and comes from in-vivo and in-vitro studies performed with different stressor (i.e. Rotenone, MPTP or proteasome inhibitors) and post-mortem human evidences in PD patients supporting a causative link between the two key events. Temporal, effect and dose concordance was established in a number of experiments (Fornai et al. 2005; Fornai et al. 2003; Betabret et al. 2000 and 2006).
KE4 => KE5 Degeneration of DA neurons of nigrostriatal pathway leads to neuroinflammation	MODERATE	Rationale: multiple in vivo and in vitro experiments support the link between degeneration of DA neurons in the nigrostriatal pathway and neuroinflammation. The observation of concomitant presence of reactive microglial and astrocytic cells and degenerated/degenerating DA neurons is also reported in many studies with a good temporal and response concordance.
KE5 => KE6 Neuroinflammation leads to degeneration of DA neurons of nigrostriatal pathway parkinsonian motor symptoms	MODERATE	Rationale: multiple in vivo and in vitro experiments support the link between neuroinflammation and degeneration of DA neurons in the nigrostriatal pathway. The observation of concomitant presence of reactive microglial and astrocytic cells and degenerated/degenerating DA neurons is also reported in many studies with a good temporal and response concordance. Neuroinflammation has been implicated in dopaminergic neuronal cell death in PD patients (Vivekanantham et al., 2014).
KE6 => AO Degeneration of DA neurons of nigrostriatal pathway leads to parkinsonian motor symptoms	STRONG	Rationale: The experimental support linking the degeneration of DA neurons of nigrostriatal pathways with the manifestation of motor symptoms of PD comes from human in vivo observations as well as from monkey, mice and rat in vivo models exposed to an experimental toxin ie rotenone and MPTP. Observations in human allow defining correlation between the levels of striatal DA with the onset of motor dysfunction (Lloyd et al. 1975; Hornykiewicz et al. 1986; Bernheimer et al. 1973). Temporal, effect and dose concordance comes from studies performed in multiple animal species following administration of rotenone and MPTP (Bezard et al. 2001; Cannon et al. 2009; Petroske et al. 2001; Alvarez-Fischer et al. 2008; Blesa et al. 2012; Lloyd et a. 1975).

Uncertainties and Inconsistencies

- There is no strict linear relationship between inhibitor binding and reduced mitochondrial function. Low doses of rotenone that inhibit CI activity partially do not alter mitochondrial oxygen consumption. Therefore, bioenergetics defect cannot account alone for rotenone-induced neurodegeneration. Instead, under such conditions, rotenone neurotoxicity may result from oxidative stress (Betarbet et al., 2000). Few studies used human brain cells/human brain mitochondria. Therefore, full quantitative data for humans are not available.
- It is molecularly unclear how rotenone binding alter CI function, switching it to ROS production. It is still unclear whether the site of superoxide production in CI inhibited mitochondria is complex I itself or not (Singer and Ramsay, 1994).
- Some studies suggest that rotenone and MPTP may have effects other than CI inhibition, e.g. MPTP and rotenone can induce microtubule disruption (Feng, 2006; Ren et al., 2005Cappelletti et al., 1999; Cappelletti et al., 2001, Brinkley et al., 1974; Aguilar et al. 2015).
- There are additional feedback possible between KEs, e.g. ROS production from KE2 may damage CI, this leads to enhancement of KE1.

Some KEs e.g. KE 2, 3, 5 pool molecular processes that may need to be evaluated individually at a later stage.

- The exact molecular link from mitochondrial dysfunction to disturbed proteostasis is still unclear (Malkus et al 2009; Zaltieri et al. 2015).
- The role of ATP depletion vs. other features of mitochondrial dysfunction is not clear.
- The role of a α-synuclein in neuronal degeneration is still unclear as well as the mechanisms leading to its aggregation.
- It is not clear under which conditions KE3 and KE5 become modulatory factors, and when they are essential. MPTP can induce damage to nigrostriatal neurons without formation of Lewy bodies (Dauer 2003; Forno 1986, 1993). Similarly, discontinuous administration of rotenone, even at high doses, damages the basal ganglia but produce no inclusions (Heikkila et al. 1985; Ferrante et al. 1997, Lapontine 2004). To reproduce the formation of neuronal inclusions, continuous infusion of MPTP or rotenone is necessary. Acute intoxication with rotenone seems to spare dopaminergic neurons (Dauer et al 2003, Ferrante 1997). In addition, in rats chronically infused with rotenone showed a reduction in striatal DARPP-32-positive, cholinergic and NADPH diaphorase-positive neurons (Hoglinger 2003) or in other brain regions. These results would suggest that Rotenone can induce a more widespread neurotoxicity (Aguilar 2015) or the model is not reproducible in all laboratories.
- Inconsistent effects of MPP+ on autophagy (up or down regulation) are reported (Drolet et al., 2004: Dauer et al., 2002). There is conflicting literature on whether increased autophagy would be protective or enhances damage. Similarly, a conflicting literature exists on extent of inhibition or activation of different protein degradation system in PD and a clear threshold of onset is unknown (Malkus et al. 2009; Fornai et al. 2005).
- The selective vulnerability of the SN pc dopaminergic pathway does not have a molecular explanation.
- Priority of the pattern leading to cell death could depend on concentration, time of exposure and species sensitivity; these factors have to be taken into consideration for the interpretation of the study's result and extrapolation of potential low-dose chronic effect as this AOP refers to long-time exposure.
- The model of striatal DA loss and its influence on motor output ganglia does not allow to explain specific motor abnormalities observed in PD (e.g. resting tremor vs bradykinesia) (Obeso et al. 2000). Other neurotransmitters (Ach) may play additional roles. Transfer to animal models o PD symptoms is also representing an uncertainties.
- There are some reports indicating that in subacute rotenone or MPTP models (non-human primates), a significant, sometimes complete, recovery of motor deficits can be observed after termination of toxicant treatment. The role of neuronal plasticity in intoxication recovery and resilience is unclear.
- This AOP is a linear sequence of KEs. However, mitochondrial dysfunction (and oxidative stress) and impaired proteostasis are influencing each other and this is considered an uncertainties (Malkus et al. 2009).

Quantitative Consideration

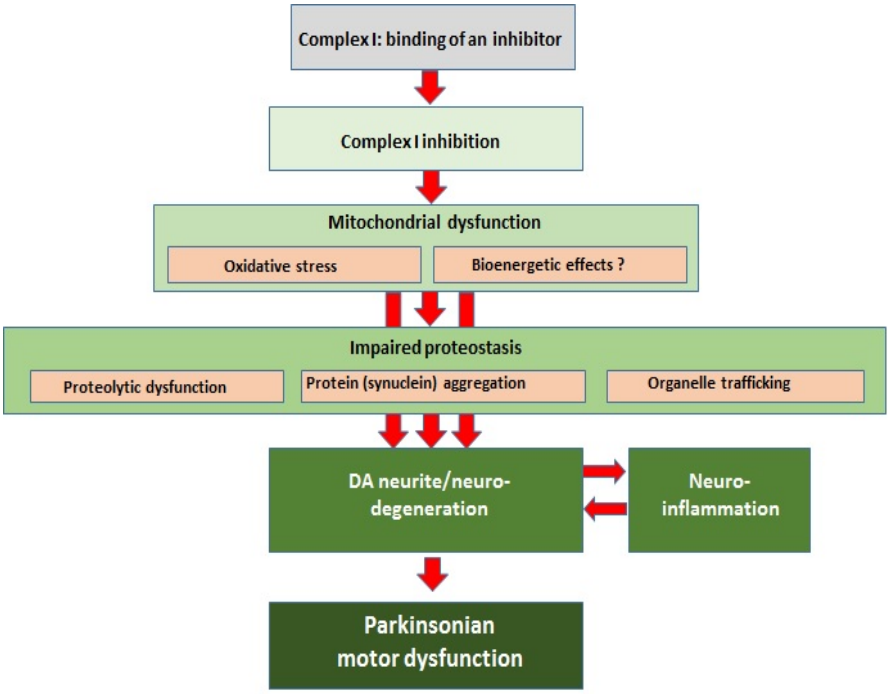
The quantitative understanding of this AOP includes a clear response-response relationship and the identification of a threshold effect. The WoE analysis clearly supports the qualitative AOP as a means to identify and characterize the potential of a chemical to induce DA neuronal loss and the AO. Importantly, both the AO and the KE4 are considered relevant regulatory endpoints for this AOP. The empirical evidence supports existence of a response-response relationship. This response-response is likely triggered by a the brain concentrations of approximately 20-30 nM and 17-47 µM of rotenone and MPP+ respectively and both concentrations trigger approx. a 50% inhibition of mitochondrial complex I and this could be considered as a "threshold". However, a more detailed dose-response analysis for each KE is lacking as well as it is not clear which temporal relationship exists for lower CI inhibitory effects. It is clear from the analysis of the AOP that for the identification of these AOs, the design of the in-vivo studies should be tailored as to a MIE which leads to a long-lasting perturbation of the KEs. This provides the most specific and definite context to trigger neuronal death. To observe KEs relevant for this AOP, new endpoints need to be introduced. Although a dose, response and temporal relationship is evident for most KEs, the quantitative relationship between impaired proteostasis and degeneration of DA neurons has yet to be elucidated. Moving from a qualitative AOP to quantitative AOP would need a clear understanding of effect thresholds and this is still representing a major hurdle for several KEs of this AOP.

Table 3 Summary of quantitative effects at the concentration of rotenone and MPTP triggering the AO

Concentration	KE1 Inhibition of C I	KE2 Mitochondrial dysfunction	KE3 Impaired proteostasis	KE4 Degeneration of DA neurons of nigrostriatal pathway	AO Parkinsonian motor symptoms
Rotenone 20-30 nM rat brain concentration [1-2]	Approx. **53%**[4-5]	Approx. 20-53% (decrease in respiration rate)[1-2]	Approx. 20-60% (decrease in UPS (26S) activity) [3]	Neuronal loss (50% of animal affected) [2]	Motor impairment (100% of animals with neuronal loss) [2]
MPP+ 12-47 µM rat brain concentration [4-5]	Approx. **50-75%** [5]	Approx. 38% (reduction in phosphorylating respiration) [5]	Approx. 60% (decrease in UPS activity) [4]	Approx. 50% of neuronal loss [4-5]	Motor impairment [4]

[1]; Okun et al. 1999 [2]; Barrientos and Moraes 1999; [3] Borland et al.2008 [4] Thomas et al 2012; [5] Betarbet et al 2000 and 2006.

Summary of the proposed Key Events in this AOP:



(https://aopwiki.org/wiki/index.php/File:Final_graph.jpg)

Chronic, low level of exposure to environmental chemicals that inhibit complex I could result in mitochondrial dysfunction and oxidative stress, triggering proteasomal dysfunction strongly implicated in parkinsonian disorders, including aggregation/modifications in α-synuclein protein and organelles trafficking. These cellular key events cause DA terminals degeneration in striatum and progressive cell death of DA neurons in SNpc, accompanied by neuroinflammation that potentiates neuronal cell death, finally leading to parkinsonian's motor symptoms. Important to notice that at each step, the effects become regionally restricted such that systemic complex I inhibition eventually results in highly selective degeneration of the nigrostriatal pathway.

Considerations for Potential Applications of the AOP (optional)

This AOP has been developed in order to evaluate the biological plausibility that the adverse outcome i.e. parkinsonian motor deficits, is linked to a MIE that can be triggered by chemical substances i.e. pesticides and chemicals in general. The relevance of the AOP has been documented by tools compounds known to trigger the described AOP. By means of using a human health outcome that has been shown in epidemiological studiesto be association with pesticide exposure, the authors intend to draw attention on this AO in the process of hazard identification. This AOP can be used to support the biological plausibility of this association during the process of evaluation and integration of the epidemiological studies into the risk assessment. It is biologically plausible

that a substance triggering the pathway, can be associated with the AO and ultimately with the human health outcome, pending the MoA analysis. In addition, this AOP can be used to support identification of data gaps that should be explored when a chemical substance is affecting the pathway. Moreover, the AOP provides a scaffold for recommendations on the most adequate study design to investigate the apical endpoints. It is important to note that, although the AO is defined in this AOP as parkinsonian motor deficits, degeneration of DA neurons is already per se an adverse outcome even in situations where it is not leading to parkinsonian motor deficits, and this should be taken into consideration for the regulatory applications of this AOP. The MIE and KEs identified in this AOP could serve as a basis for assays development that could contribute to an AOP informed-IATA construction which can be applied for different purposes such as: screening and prioritization of chemicals for further testing, hazard characterization or even risk assessment when combined with exposure and ADME information.

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