EXTERNAL REVIEW REPORT

AOP-3

Title: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to Parkinsonian motor deficits

Introduction and Background

Currently, millions of people, especially those over the age of 60, suffer from progressive neurodegenerative diseases such as Parkinson's. The AOP 3 describes the linkage between inhibition of complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial respiratory chain and motor deficit as observed in Parkinsonian disorders. A molecular initiating event (MIE) is inhibition of mitochondrial complex I and an adverse outcome (AO) is symptoms like Parkinsonian disorders, including Parkinson's disease (PD). This AOP includes the stressors rotenone and MPTP, key events (including mitochondrial dysfunction, impaired proteostasis, neuroinflammation and degeneration of dopaminergic neurons of the nigra-striatal pathway) and multiple key event relationships. Information from *in vitro* and *in vivo* experiments is further substantiated by human studies in brain tissues from individuals with sporadic PD to support the pathways of toxicity proposed in this AOP. This AOP will have applications in neurotoxicity assessment of chemicals that structurally resemble rotenone and MPTP, which may inhibit mitochondrial complex I leading to degeneration of nigra-striatal dopaminergic neurons and adverse outcome Parkinsonian disorders.

External reviewers: Cristina Sunyol, Yen-Ching Wu, and Anna Forsby

Authors: Anna Bal-Price, Marcel Leist, Stefan Schildknecht, Florianne Tschudi-Monnet, Alicia Paini, and Andrea Terron

Main Issues/Comments (here onwards "comment") of AOP External Reviewers are summarized below under the four specific charged questions. Below each question is the authors' response (marked in *italics*):

Charged question 1: *Check if the AOP incorporates the critical scientific literature and if the scientific content of the AOP reflects the current scientific knowledge on this specific topic*

All reviewers, in principle, agree that the AOP is very thorough as it incorporates current scientific knowledge in the context of chemical (rotenone, MPTP and its metabolite MPP+, or paraquat)-induced mitochondrial complex I inhibition in nigra-striatal discrete brain region leading to neurodegeneration of the dopaminergic pathway and Parkinsonian motor disorders. Reviewers are very complimentary that the review of the MIE, the 6 KEs, the KERs and the AO

is thorough and most conclusions are supported by current knowledge. Reviewers, however, have raised some issues, which are as follows:

Comment-1: The MIE needs to be better documented with additional references (Page 4), as in the present form the substantive literature for binding of MPP+ at the mitochondrial complex I is sparse. The reference of Schuler F and Casida JE (Functional coupling of PSST and ND1 subunits in NADH: Ubiquinone oxidoreductase established by photoaffinity labelling. *Biochim et Biophys Acta* 1506: 79-87, 2001) is suggested for citation to support the photoaffinity labelling approach to establish that rotenone (2uM) and MPP+ (20 mM) inhibit the binding of [^{3}H]TDP to the PSST subunit of complex I in electron transport particles. On page 3 of the AOP SNAPSHOT, the reviewers have also pointed out that the cited reference (Cleeter *et al*., 1992. Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyrdinium: evidence for free radical involvement. *J. Neurochem*. **58**: 786-789) corresponds to the KE1 (Inhibition of NADH-ubiquinone 1 reductase) rather than to the MIE.

Response: *The references of Schuler F and Casida JE (Functional coupling of PSST and ND1 subunits in NADH: Ubiquinone oxidoreductase established by photoaffinity labelling. Biochim et Biophys Acta 1506: 79-87, 2001) and Fendel et al. (2008) were added as additional references to the MIE. Cleeter et al. (1992) was removed from the MIE section and moved to the KE1 as suggested. In the text, "How this key event works", the authors added "Prolonged treatment with an inhibitor results in a severe, progressive and irreversible inhibition of complex I, most likely by indirect mechanisms involving oxidative damage (Cleeter et al., 1992)". The reviewers agreed that the response and the actions provided by the authors were adequate and no further actions are deemednecessary.*

Comment-2: References are needed to support the suggested explanation as to why dopaminergic (DA) neurons projected from the substantia nigra pars compacta (SNpc) are sensitive to rotenone and MPTP, but the DA neurons in the ventral tegmentum area (VTA) are insensitive to the stressors (Page 23). The reviewers requested an estimated certainty for this explanation. For instance, are heart muscle cells that also express Ca v1.3 L-channels and are strongly dependent on mitochondrial function also more sensitive to rotenone than other cells?

Response: *The prefrontal cortex is a primary site of projection for VTA dopaminergic neurons, so this difference may be of importance in understanding the relative resistance of VTA neurons to PD-related degeneration. Differences in neuronal milieu have also been identified surrounding SNpc dopaminergic cell bodies. The neuropil of the substantia nigra, composed of axon projections from the striatum and globus pallidus, stains strongly for calbindin D_{28K}, and most dopaminergic cell bodies reside within this calbindin-rich neuropil (Damier et al., 1999a; Dauer, 2003; Fujita et al., 2014).*

Rotenone produces acute neurotoxicity by inhibiting the activity of mitochondrial respiratory complex I, and inducing cell death by apoptosis due to excess generation of free radicals. Common clinical signs of poisoning include nausea, vomiting, gastric pain, clonic convulsions,

muscle tremors, lethargy, incontinence and respiratory stimulation followed by depression. Death occurs due to cardio-respiratory failure (Gupta R. 2012. Rotenone. In Veterinary Toxicology: Basic and Clinical Principles. Chapter-52, pages 620-623). Indeed, cardiac toxicity is the toxicity limiting dose also in experimental conditions using rotenone as a chemical tool to reproduce PD-like lesions in SNpc (Betarbet et al., 2000). During the TC discussion, the reviewers agreed that the response provided by the authors was adequate and no further actions are deemed necessary. The text is amended accordingly under the description of the KE4.

Charged question 2: ***Verify the weight of evidence judgement/scoring provided by AOP developers for KEs, KERs and the overall AOP***

The reviewers, in general, agreed with the authors' assessment of WOE, KERs and essentiality of the KEs, as they are well documented. However, the reviewers noted a lack of very specific literature for some KEs and KERs. Some of the main issues are as follows:

Comment-1: KER: Neuroinflammation leads to degeneration of DA neurons of the nigra-striatal pathway, and the reversal KER: Degeneration of DA neurons of the nigra-striatal pathway leads to neuroinflammation (Pages 56 and 64). Authors need to provide evidence of whether paraquat is an inhibitor of complex I or complex III, or both, so a link with the MIE or with downstream KE/KEs of this AOP could be established.

Response: *The authors agreed with the reviewers and the stressor paraquat is removed from the AOP. Paraquat was used by the authors to develop an additional AOP which is not yet submitted to Wiki, where the late KES are in common with this AOP.*

The authors explained to the reviewers that paraquat was initially used as a stressor but during the development of the AOP it became clear that paraquat was not an adequate stressor to describe the empirical support for the MIE and the KE1 and their relationship. The authors explained that a different AOP was built using paraquat as a stressor and that some later KEs will be common to the two AOPs. Most of the empirical support provided by paraquat will be removed from this AOP. The reviewers agreed with the proposal and with the explanation given by the authors and no further actions are deemed necessary.

Comment-2: In the Overall Assessment of the AOP (Page 99 onwards and in the Table), the reviewers noted that KE4 and KE6 are identically named, i.e. "Degeneration of DA neurons of nigra-striatal pathway", and reviewers accordingly requested the authors/developers of the AOP to clarify this issue.

Response: *The KE "Degeneration of DA neurons of nigra-striatal pathway" is quoted twice in this AOP as the KE relationship between "Degeneration of DA neurons of nigra-striatal pathway" and the KE "Neuroinflammation" is intended to cover two different mechanisms. This is because "Neuroinflammation" can be both the cause as well as the consequence of "Degeneration of DA neurons of nigra-striatal pathway". This was necessary in order to describe the double arrow linking "Degeneration of DA neurons of nigra-striatal pathway" to "Neuroinflammation" in the graphical description. To do this in the AOPWiki, and to keep the*

two mechanisms separate (i.e. two different KERs are linking the same KEs) the authors had to include the KE "Degeneration of DA neurons of nigra-striatal pathway" twice. The authors explained to the reviewers that changing the name of the KE will also have important implications for the AOP network as the new name will be not recognized. This technical solution was recommended by the Wiki manager and came after discussion with members of the EGMAST group.

Comment-3: The reviewers refuted that the contents of the sentence "However, as a recovery (of tyrosine hydroxylase) is possible, this may not be associated with irreversible degeneration and is a signal sufficient to trigger microglial reactivity" in the description of KE4 (Page 100) are supported by the cited reference (Sandström *et al*., 2014).

Response: *The authors agreed with the reviewers and the essentiality table for the KE4 was reconsidered and the following test proposed. Receptors for AGEs can activate NF-kB (a transcription factor involved in the inflammatory response) and they are found on microglia cells and astrocytes. Ablation of RAGE proved to be protective against MPTP-induced decreases of TH^+ neurons and mitigation of microglia and astrocytes reactivity was observed (Teismann et al. 2012). Inhibition of RAGE, which is upregulated in the striatum following rotenone exposure and in response to neuroinflammation, decreases rotenone-induced apoptosis by suppressing NF-kB activation, as well as the downstream inflammatory markers TNF-alpha, iNOS and myeloperoxidase (Abdelsalam and Safar, 2015). This showed intermingled links between neuronal injury/death and neuroinflammation. Rotenone-induced neurotoxicity was less pronounced in neuron-enriched cultures than in neuron-glia co-cultures (Gao et al., 2002), suggesting that neuron-glia interactions are critical for rotenone-induced neurodegeneration. In addition, in in vitro systems, a decrease in TH mRNA expression has been observed to be a sufficient signal to trigger microglial reactivity (Sandström et al., 2017). The reviewers agreed with the author's proposal and no further actions were deemed necessary. The reviewers made a recommendation that all abbreviations in the AOP should be spelled out at their first occurrence. The authors agreed and the amended text is reflected in the Wiki.*

Comment-4: KER: Mitochondrial dysfunction I leads to degeneration of dopaminergic neurons of the nigra-striatal pathway (Page 67 onward). Information on the empirical support is based on oxidative stress and ATP reduction, but the information on degeneration of DA neurons of the nigra-striatal pathway is scarce. Furthermore, empirical support for the data, specifically the degeneration of the nigra-striatal neurons, needs to be provided.

Response: *The authors thanked the reviewers for this essential aspect of the AOP. They included the following empirical support highlighting the degeneration of nigra-striatal DA neurons as a consequence of complex I inhibitor dependent mitochondrial dysfunction (added to KER 7):*

- *Humans + MPTP. Neuropathological examination reveals severe depletion of neurons of the SNpc, no Lewy bodies, clustering of glia; indications for self-perpetuating*

neurodegeneration following initiation by MPTP. Patients display Parkinsonian motor deficits; responsive to L-Dopa (Ann Neurol 1999, 46(4), 588-605). Nigrostriatal DA neurodegeneration confirmed in live patients by PET analysis (Ann Neurol 1994, 36(5), 765-770).

- *Mice/rats + MPTP. Intrastriatal infusion of MPP^+. EC_{50} for DA neurotoxicity was 0.4 mM (mice) and 4.3 mM (rats). Depletion of striatal DA reflected this ca. 10-fold difference between mice and rats (JPET 1994, 270(3), 1008-1014).*

- *Rats/intranasal infusion of MPTP (0.1 mg/nostril). Reduction in DA neurons in the olfactory bulb and the substantia nigra by ca. 50 %. Reduction in DA by ca. 50 %. Correlation of nigra-striatal DA neuron loss with the onset of impaired motor functions (Exp Neurol 2006, 202(2), 391-403).*

- *Rats + intranasal MPTP. Decrease in TH staining in the olfactory bulb and SNpc by ca. 30 %. Reduction of DA by ca. 50% in olfactory bulb and 25 % in striatum. Correlated with olfactory, cognitive, and motor impairments (Ann NY Acad Sci 2009, 1170, 629-636).*

- *Mice + MPTP. Strain differences in the response towards MPTP. Comparison of C57/bl vs. CD 1 mice. C57/bl mice displayed 90 % reduction in striatal DA, while CD1 mice exhibited a reduction of only 30 %. TH expression levels reduced by 45 % in C57/bl mice vs. 27 % reduction in CD1 mice (Exp Neurol 1994, 126(2), 195-204).*

- *Mice + MPTP. Mice lacking DAT are resistant towards MPTP. Loss of TH-positive neurons in the nigrostriatal system by 34 % in wt (+/+) mice; loss of 22.5% in DAT (+/-) mice; loss of 0 % in DAT (-/-) mice (Exp Neurol 1999, 155, 268-279).*

- *Rats + rotenone. Loss of striatal fibers (54 %), loss of nigral neurons (28.5 %). TH staining intensity reduced by 50 % in the striatum, reduced by 70 % in the substantia nigra (J. Neurochem 2003, 84, 491-502).*

- *Rats + rotenone (low 1.5 mg/kg/day vs. high 2.5 mg/kg/day) Dose-dependent loss of striatal DA, loss of TH immunoreactivity in the striatum; dose-dependent catalepsy (Behav Brain Res 2002, 136, 317-324).*

- *C57/bl 6 mice + rotenone. Oral administration of rotenone for 28 days leads to nigra-striatal DA neurodegeneration, correlation with the onset of motor deficits (J. Neurochem 2007, 101, 1491-1504).*

The authors added additional literature in the AOP under the dedicated KER and the reviewers agreed to this proposal. The reviewers commented that the updated list of references was comprehensive enough for the empirical support of the KER.

Comment-5: KER: Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I) leads to inhibition, NADH-ubiquinone (Page 85). The reviewers suggested inclusion of the data on inhibition of NADH-ubiquinone activity from the works of Ramsay *et al* (1991) and Schuler and Casida (2001). The reviewers have suggested that this should also be included in Overall assessment of the AOP.

Response: *As suggested by the reviewers, the two publications are now included at the end of the text in empirical support for linkage in this KER:*

- *MPP+ interrupts mitochondrial electron transfer at the NADH dehydrogenase-ubiquinone junction, as do the respiratory chain inhibitors rotenone, piericidin A and barbiturates. The 4'-alkyl derivatives of MPP+ inhibit NADH oxidation in submitochondrial particles at much lower concentrations than does MPP+ itself. The MPP+ analogues with short alkyl chains prevent the binding of [^{14}C] piericidin A to the membrane and thus must act at the same site, but analogues with alkyl chains longer than heptyl do not prevent binding of [^{14}C]piericidin (Ramsay et al., 1991).*
- *13 complex I inhibitors were found to decrease labelling at the PSST site without effect on ND1 labelling. The results suggest that the common action of MPP+ and stigmatellin on the functional coupling of the PSST and ND1 subunits is initiated by binding at a semiquinone binding site in complex I (Schuler and Casida, 1991). The authors have also included two additional publications in the reference list.*

Ramsay RR, Krueger MJ, Youngster SK, Singer TP. (1991) Evidence that the inhibition sites of the neurotoxic amine 1-methyl-4-phenylpyridinium (MPP+) and of the respiratory chain inhibitor piericidin A are the same. Biochem J 273(Pt 2):481-4.

Schuler F, Casida JE. (2001) The insecticide target in the PSST subunit of complex I. Pest Manag Sci 57(10):932-40.

The reviewers agreed with the author's response. In addition, the references are included in the "empirical support for the overall assessment of the AOP" in the Wiki.

Comment-6: The reviewers identified the strength of the AOP, i.e., it is very well organized and that the KERs are sound. The reviewers, however, highlighted a weakness that this AOP is based on rotenone, MPTP (MPP+, metabolite of MPTP), and paraquat that bind at mitochondrial complex I, and there is no information on whether other mitochondrial complex I inhibitors also perturb the KEs (specifically degeneration of DA neurons in the SNpc) or produce a similar AO. In other words, this AOP appeared not to be applicable to all inhibitors of mitochondrial complex

I. The reviewers suggested that the authors check the contents of Fato *et al* (2009), Esposti *et al* (1993), Lagoa *et al* (2011), and Park *et al* (2003), and include these papers in the revision of the AOP. Furthermore, the authors speculated on Page 102 that kinetic features may counteract the AO and this needed to be supported by a reference(s).

Responses:

Part 1: *The reviewers addressed one of the most fundamental aspects of the present AOP, as the MIE is defined as an event independent of the respective compound applied for its inhibition, i.e., chemically agonistic. The vast majority of empirical support available in the literature is based on complex I inhibitors, such as rotenone and MPTP/MPP+, as well as on studies involving genetic impairment of complex I activity. A relatively wide spectrum of structurally different complex I inhibitors have been described over the course of recent decades. Prominent examples are acetogenins (Nat Prod Rep 2005, 22, 269-303); alkaloids (J Neurochem 1996, 66, 1174-1181); antibiotics (BBA 1998, 1364, 222-235; Eur J Biochem 1994, 219, 691-698; JBC 1970, 245, 1992-1997; Bioorg Med Chem 2003, 11, 4569-4575); pesticides (Biochem Soc Trans 1994, 22, 230-233); quinones (JBC 1971, 246, 2346-2353); or vanilloids (ABB 1989, 270, 573-577).*

All of these structurally different complex I inhibitors were characterized with isolated mitochondria or with submitochondrial particles. Application of bovine heart mitochondria revealed IC_{50} values in the range of 20-70 nM for piericidin A, fenpyroximate, rotenone, and phenoxan (Eur. J. Biochem 1994, 219, 691-698). IC_{50} values in the range of 1-10 nM were detected by application of submitochondrial particles with rotenone, molvizarin, rollinstatin-1 and -2, and piericidin A (Biochem J. 1994, 301, 161-167).

Studies involving neuronal cell cultures or in vivo models are in fact rather rare. A systematic comparison of the IC_{50} values for complex I inhibition and EC_{50} values for the reduction of ATP levels; cell death was performed with rat fetal striatal neurons (Exp Neurol 2009, 220, 133-142). Due to the lipophilicity of most of the complex I inhibitors tested, the detected EC_{50} values were in most cases lower than the IC_{50} values detected for complex I inhibition. EC_{50} values detected were: annonacin (60 nM), fenazaquin (45 nM), piericidin A (1.6 nM), rollinstatin- 2 (1 nM), rotenone (8 nM), and squamocin (1 nM).

A systematic investigation involving mesencephalic cultures as well as rats was performed for the complex I inhibitor annonacin, a major acetogenin of soursop, a plant suspected to cause an atypical form of PD in Guadeloupe. Mesencephalic cultures treated for 24 h with annonacin revealed EC_{50} values of 20 nM (annonacin, 34 nM (rotenone), and 1900 nM (MPP^+) (Neurosci 2003, 121(2), 287-296). Intravenous application by minipumps over the course of 28 days indicated a passage of annonacin across the blood-brain barrier, and an energy-dependent loss of ca. 30 % of DA neurons in the substantia nigra (J Neurochem 2004, 88, 63-69).

The authors indicated that they are aware of this limitation which is actually summarized in the overall weight of evidence (strength, consistency, and specificity of association of AO and MIE).

The authors have included the above text in the same section of the AOP and briefly described these limitations as uncertainties. The authors complimented the reviewers for their suggestions.

The reviewers commended the authors for the comprehensive answers given during the TC discussion and agreed with the responses offered by the authors to include the references provided and a summary of the text included under the sections on "specificity of the AOP" and "Uncertainties" for the overall assessment of the AOP.

Part 2:

The AOP has not included TK data as part of the conceptual framework which is chemically agonistic and potentially applicable to any chemicals. External exposure to target site (TK) is covered by the MOA conceptual framework which is chemical specific. AOP has indeed covered the TD part of the pathway. Consequently, when dealing with specificity of the pathways from MIE to the AO the authors assumed that the compound acts at the target site, but availability and/or understanding of TK data/behavior is highly relevant for the interpretation of the empirical support and features such as uptake, metabolism, and excretion which can have significant influence on the toxicity of different complex I inhibitors; including the ability of a chemical stressor to reach the target site. These aspects, for example, are of particular relevance with respect to MPTP, as this pro-toxicant requires activation to form the active metabolite MPP+ at the target site (Antiox Redox Signal 2015, 23(13), 1001-1016). Clearance of MPTP and its metabolites was significantly different in rats and mice (JPET 1988, 244(2), 443-448). It was further observed that extracellular MPP+ clearance from the brain is completed after 1-2 h following MPTP intoxication (Neurotox Res 2014, 25, 135-145). Furthermore, MPP+ can be sequestered by non-neuronal cells by Oct-3-dependent uptake and hence influence MPP+ toxicity in DA neurons (J Neurochem 2003, 85, 358-367). These aspects need to be considered in the interpretation of data obtained with different complex I inhibitors. The above cited literature is included in the AOP. The sentence mentioned by the reviewer was added as a commentary to explain that TK data should be taken into account to explain the limitation of available stressors able to support the late KEs. For clarity, the authors amended the sentence to "When considering the limited amount of chemical stressors for which empirical data are available for supporting the full sequence of KEs, kinetic and metabolic considerations should be taken into account to demonstrate specificity for these compounds", and included it in the text under the"specificity" of the AOP.

Comment-7: The reviewers asserted that the published conflicting data (Choi *et al*. 2008. Mitochondrial complex I inhibition is not required for DA neuron death induced by rotenone, MPP+, or paraquat. *PNAS* 105(39), 15136-15141) challenges the current AOP. The cited research article shows that abolishment of complex I's activity by inactivation of a gene that codes for a subunit of complex I does not impact the survival of DA neurons in culture. The actions of rotenone, MMP+ or paraquat are independent of complex I. Since some complex I

inhibitors also target other complexes, it is possible that impairment of other respiratory complexes may be involved.

Response: *The authors indicated that they cannot rule out that those off-target effects of complex I inhibitors might exist in a cell. Studies involving the expression of the inhibitor-insensitive oxidoreductase NDI-1 indicates a rescue from complex I inhibitors, hence suggesting that off-target effects are not predominantly involved, otherwise neurons would also degenerate under conditions of NDI-1 expression + inhibitor.*

In Ndufs4 (-/-) cells, an alternative electron input via complex II might compensate energy impairment. These observations would point to a dominating role of inhibitor-dependent ROS formation in the observed neurodegeneration. These aspects however remain speculative and require a detailed investigation of ATP generation and ROS formation in Ndufs4(-/-) cells and in NDI-1 expressing cells in the presence and absence of complex I inhibitors.

The reviewers agreed with the authors' response and no further actions are deemed necessary on this point. It was also pointed out during the TC discussion that the reference was quoted in the context of the empirical support for KER. It is worth noting that the AOP conceptual framework is ranking biological plausibility, essentiality and empirical support on different levels of relevance and that the biological plausibility for this KER still remains strong.

The authors also had an additional internal discussion on the paper published by Choi et al (2008) disproving a role of complex I in rotenone, and MPP+ toxicity. It was noted that this paper used the approach of genetically deleting an essential chaperone in complex I assembly, and the authors found that absence of complex I activity in this model did not affect the toxicity of rotenone and MPP+. The authors, however, noted that the findings have never been confirmed/ continued, neither in the originating laboratory, nor by others. Second, the work did not consider the possibility that some functions of complex I were not affected by the absence of the chaperone (e.g. reverse electron transfer from complex II and III), and that rotenone and MPP+ may well cause toxicity by interfering with such residual function (e.g. by favoring channeling of electrons to molecular oxygen). In light of this situation, the publication of Choi et al (2008) should be considered weak in the weight of evidence. It was therefore considered at best a minor inconsistency. The authors included this position under the uncertainties list for the overall assessment of the AOP.

Comment-8: The reviewers emphasized that a large variability exists regarding the onset of the downstream KEs of this AOP, such as degeneration of DA neurons and Parkinsonian motor deficits. Data indicates that their presence is dependent upon the complex I inhibitor being used and the route of exposure. Using three independent scenarios (exemplified by published papers: Perez-Pardo *et al*., 2017; Minnema *et al*., 2014; Johnson and Bobrovskaya, 2015; Greenamyre *et al*., 2010; Hollinger *et al*., 2003; Champy *et al*., 2004), the reviewers suggested that the neuronal degeneration of DA neurons may not always lead to the onset of Parkinsonian motor deficits, as

proposed by the KER: Degeneration of DA neurons of the nigra-striatal pathway leads to Parkinsonian motor deficits (Page 76).

Response: *The authors agreed that different complex I inhibitors and different application protocols lead to variable patterns of DA neurodegeneration and motor deficits. The available data indicate a close correlation between striatal DA levels and the onset of Parkinsonian motor deficits. This notion is supported by the comparison of established in vivo protocols (MPTP, rotenone) that result in a reproducible degeneration of nigra-striatal DA neurons and the onset of motor deficits (Neurosci 2012, 211, 51-76). A recovery from motor impairments has been observed primarily in association with MPTP application. One explanation for these observations can be found in the variability of expression levels of DA neuron proteins that are often used as markers for DA neuron number, such as protein expression of TH, VMAT-2, or DAT. A reduction in the expression levels of these targets has been observed in the absence of cell loss. It could hence be speculated that recovery from motor deficits depends on elevated DA levels as a result of normalized protein levels of these DA-specific targets.*

The authors team thoroughly discussed the impact of different routes of exposure and differences in the experimental protocols affecting the final outcome, i.e. , Parkinsonian motor deficits. The authors agreed that different routes of administration will have an impact on the final outcome; though it was recognized that this is likely due to changes in TK. The assessment of TK is not included in the AOP conceptual framework which is chemically agonistic and the concentration of the stressor able to trigger the full cascade of KEs is intended to be at the target site. It was also noted that a drop in the incidence of the occurrence of the effect and the increase in variability in later KEs is, however, biologically plausible and this should be considered as part of the response evaluation where incidence of the effect in later KEs is expected to be lower. This is particularly important for the KER linking DA neuronal degeneration and Parkinsonian motor deficit where about 40 to 60% of DA neurons should be lost to see the clinical evidence of Parkinsonian motor deficits. However, from toxicological and regulatory perspectives, a statistically significant drop in the number of DA neurons is still considered an adverse outcome.

The authors agreed with the reviewers and have added "variability in the experimental outcome consequent to differences in the route of exposure" in the uncertainties list for this KER.

Comment-9: The reviewers made a note that some of the suggested endpoints to detect degeneration of DA neurons of the nigra-striatal pathway may not be applicable to all inhibitors (Page 22). The reviewers cited the reference of Tieu (2011) *A guide to neurotoxic animal models of Parkinson's disease*. Cold Spring Harb Perspect Med., and highlighted that while paraquat treatment damages DA neurons, its application has not consistently led to damage in DA cell bodies and terminals and may not have an effect on striatal dopamine levels. The reviewers further reiterated that the endpoints described under the heading "How it is measured or detected", to assess degeneration of DA neurons may not be applicable to all chemicals that inhibit complex I, and applying the wrong endpoints may result in erroneous negative findings.

Response: *The authors agreed with the note made by the reviewers that the endpoints described on page 22 may not be applicable to all chemical stressors acting on this pathway. The authors have added a comment under the subchapter "How this key event works" and to changed the introduction to the subchapter "How it is measured or detected". Although different animal models of PD exist, it is recognized that despite many accomplishments, current models based on chemically induced neurotoxicity still have significant shortcomings. The ability of these experimental models to accurately recapitulate the pathology, symptoms, and pathogenic mechanism as seen in PD patients has a number of limitations and consequently some of the endpoints described here to detect degeneration of DA neurons of the nigra-striatal pathway may not be applicable to all chemical stressors. For example, although the total density of striatal dopaminergic terminals frequently correlates with the number of their cell bodies in the substantia nigra, it is not uncommon to observe differential damage or protection between these two structures. With paraquat, the dopaminergic cell bodies are more vulnerable than their terminals. On the other hand, striatal terminals are more sensitive to methamphetamine, MPTP, or 6-OHDA toxicity. Quantifying striatal dopaminergic terminals is therefore also informative (Tieu, 2011).*

Changes are incorporated under the "How it is measured or detected" introduction on page 23. Depending on the chemical stressor, study design and animal species/strain, the methodology required for the analysis of neuropathology and function may vary as it is recognized that different endpoints can be quantitatively and/or qualitatively affected differently. However, when testing potential neurotoxic chemicals, it is important to assess the integrity and function of the nigra-striatal pathway. Some well-developed and accepted techniques that are commonly used for these purposes are described (Tieu, 2011): Quantification of dopaminergic neurons in the substantia nigra par compacta, quantification of dopaminergic terminals in the striatum, quantification of dopamine content in the striatum and detection of Lewy Body-like aggregation. During the TC discussion, the reviewers agreed with the author's response and no further actions are deemed necessary.

Charged question 3. ***What would be the regulatory applicability of this AOP in your opinion?***

The reviewers made a note of three points, which are as follows:

1. The reviewers suggested applicability of this AOP for neurotoxicity assessment, since it is plausible that a compound that binds to the mitochondrial complex I may eventually lead to Parkinsonian motor deficits.

2. The reviewers pointed out that the regulatory applicability of this AOP would be to use experimental findings in model systems representing the MIE and KEs as indicators/alerts for the AO. Risk assessment may be possible if bioavailability at the target cells can be estimated, the toxic concentrations *in vitro* can be extrapolated to *in vivo* and exposure scenarios can be simulated.

3. The reviewers foresaw the applicability of this AOP for chemicals that have structural similarities to rotenone or MPTP. However, this AOP may not be useful for chemicals that do not resemble rotenone or MPTP.

Response: The authors have added points 1 and 2 in the AOP chapter on "application of this AOP", as suggested by the reviewers. The authors also agreed to include the third suggestion with a slightly different perspective. This AOP can be applied for chemicals that have structural similarities to rotenone or MPTP. However, this AOP may not at the moment be used for chemicals that do not resemble rotenone or MPTP. It is however expected that compounds acting on the same MIE, but belonging to different chemical classes and those that are structurally different, will be tested in the near future in order to substantiate a broader specificity for this AOP. However, it remains evident that chemicals affecting the MIE are potential risk factors for this AO.

Charged question 4. ***Overall assessment of the AOP***

Comment-1: The reviewers highlighted the scientific quality and the strong weight of evidence of this AOP, but also recognized the limitation of using only a few chemicals (rotenone, MPTP, and paraquat). Reviewers pointed out that there are compounds that inhibit complex I and affect mitochondrial function, but do not degenerate DA neurons in the nigra-striatal pathway. Therefore, which other chemicals bind to complex I and will or will not produce a similar AO, remains a challenge.

Response: *The authors completely agreed with this comment from the reviewers. The authors addressed this point in the uncertainties list. The authors acknowledged that this limitation already triggered important actions and that complex experimental work is actually in progress to expand the specificity of this AOP. It is expected that this AOP will consequently be updated as soon as additional information becomes available.*

Comment-2: In contrast to the opinion of internal review which recommended splitting general KEs into more specific KEs, the opinion of external reviewers was that the present KEs were in line with recent reviews that link the control of mitochondrial quality and the etiology of Parkinson's disease. The reviewers further reiterated that the KER between KE1 and KE2 is strong as well as the KER between KE2 and KE4; and splitting KE2 to more specific KEs, e.g., ATP depletion or ROS production, would make the KERs weaker because the temporal sequences are not clear.

Response: *The authors agreed with the reviewers.*

The reviewers suggested inclusion of additional references:

Fato et al. (2009) Differential effects of mitochondrial complex I inhibitors on production of reactive oxygen species. *Biochim Biophys Acta* **1787**(5): 384-392.

Esposti et al. (1993) Complex I and Complex III of mitochondria have common inhibitors acting as ubiquinone antagonists. *Biochem Biophys Res Commun* **190**(3): 1090-6.

Lagoa et al. (2011) Complex I and cytochrome *c* are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. *Biochimica et Biophys Acta* **1807**: 1562-1572.

Park C.et al. (2003) Quercetin protects the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. *Biochem Pharmacol* **66**(7): 1287-1295.

Choi et al. (2008) Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. *PNAS* **105**(39): 15136-15141.

Perez-Pardo et al. (2017) Gut-brain and brain-gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet. *Nutri Neurosci,* 2017: 1-2.

Minnema et al. (2014) Dietary administration of paraquat for 13 weeks does not result in a loss of dopaminergic neurons in the *substantia nigra* of C57BL/6J mice. *Regul Toxicol Pharmacol* **68**(2): 250-258.

Johnson M and Bobrovskaya L (2015) An update on the rotenone models of Parkinson's disease: Their ability to reproduce the features of clinical disease and model gene-environment interactions. *NeuroToxicology* **46**: 101-116.

Greenamyre et al. (2010) Lessons from the rotenone model of Parkinson's disease. *Trends Pharmacol. Sci* **31**(4): 141-2.

Höglinger et al. (2003) Chronic systemic complex I inhibition induces a hypokinetic multisystem degeneration in rats. *J Neurochem* **84**: 491-502.

Champy et al. (2004). Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical Parkinsonism in Guadeloupe. *J Neurochem* **88**: 63-69.

Tieu K. (2011). A guide to neurotoxic animal models of Parkinson's disease. *Cold Spring Harb Perspect Med* 1(1).

Response: *The authors agreed to add the suggested references to the AOP. The authors acknowledge that some of the references are already quoted in the AOP but in different sections. The authors have updated the AOP accordingly. The authors will check carefully if the newer literature suggested by the reviewers can actually substitute for the older literature referenced by the authors and also avoid redundancy whereever possible.*

The reviewers highlighted minor issues and editorial mistakes that need to be addressed by authors/developers:

1) Stressors: 1,2-dihydrorotenone is widely cited as a stressor. Is this because [^{3}H] 1,2-dihydrorotenone is used as a ligand for complex I? When 1,2-dihydrorotenone is cited, then rotenone or MPP+ are not cited. Provide any reason.

2) Some editing is needed. For example: i) a dot is needed after title names, before description is initiated: ii) on page 12, "tetramethylrhodamine, methyl ester" eliminate the colon; iii) what does N/A mean? Not applicable? Include the abbreviation as a footnote; iv) -Page 23. "markers specific for dopaminergic neurons such as tyrosine hydroxylase dopamine transporter (DAT)…" should be "markers specific for dopaminergic neurons such as tyrosine hydroxylase (TH), dopamine transporter (DAT)…"; v) Page 108; add on the reference list that of Sandström et al., 2014; vi) Page 79: ""15.1 7.5"; "12.1 7.1"; "1.0 0.1"; "11.9 5.2"; "50 11.6". Is something lacking (ex: +/-)?; vii) Page 2: Betarbet et al., 200; edit the year.

3) Most of the figures in this AOP and their legends are extracted from published work without modifications. Is permission from the publishers a requirement? If so, it should be requested and cited when appropriate.
4) The routes of administration must be indicated for all given doses. "Infusion" is not sufficient information (e.g. on page 32 and in Table 2 p. 102). This is important for the comparison to *in vitro* neurotoxicity, i.e. should the BBB be considered or is the dose indicated regarded as the dose at the target?
5) The description of how KE5 can be measured or detected (p.20) should be structured in the same way as the other KEs.
6) The names of specific suppliers/kits/products should be avoided in the descriptions for how KEs can be measured.

Responses: *All of these reviewers' queries are addressed by the authors in the AOPWiki. For each figure, the figure legend has "Courtesy of the author" (in parenthesis) to avoid seeking permission. Consistency in the nomenclature of the chemical stressors, such as rotenone and MPP+ is addressed. All the abbreviations are spelled out at their first occurrence, and thereafter used with consistency.*

Summary record of the teleconference

A teleconference (TC), using WebEx, was held on May 16, 2017, and the participants were the authors (Andrea Terron, Anna Ball Price, Alicia Paini, and Stefan Schildknecht) and the reviewers (Cristina Sunyol, Yen-Ching Wu, and Anna Forsby). External review manager (Ramesh Gupta) served as moderator to facilitate the TC process. During the TC, main issues were discussed point by point until both the reviewers and the authors were satisfied and all issues were resolved. The authors responded to each issue and all those responses are reflected in the AOPWiki. Overall, the outcome of the TC was very constructive, productive, and cordial. By the end of the TC, all main issues were resolved. During the TC, it was discussed and decided

that each issue was from all the reviewers and the response was from all authors to avoid redundancy.

Summary of revisions

The authors responded to the majority of the reviewers' queries prior to the TC, and to all queries during the TC. The authors' response(s) and discussion for each comment are provided next to each comment. The discussion and explanations will also be reflected in the AOPWiki. Of course, the authors stated that the AOPWiki is a living document and it can be amended at any time as additional information becomes available.

Further discussion

All issues were discussed and resolved during the TC, and there is no pending issue for further discussion.

Outcome of the external review

The reviewers worked very hard in identifying the main issues and deficiencies in the AOP draft. Prior to the TC and during the TC, authors diligently responded to each query, and all issues were resolved and the changes will be reflected in the AOPWiki.

Concluding remarks

As review manager, I would like to commend the authors/developers of this excellent AOP that interests not only toxicologists in academia, but many others in different disciplines and sectors. The authors proposed a pathway, i.e., inhibition of the mitochondrial complex I of nigra-striatal neurons leads to Parkinsonian motor deficits. Chemical stressors such as rotenone or MPTP are known to cause inhibition of mitochondrial complex I, mitochondrial dysfunction, impaired proteostasis, neuroinflammation, and degeneration of dopaminergic neurons of the nigra-striatal pathway. This AOP will have a regulatory applicability for those chemicals which resemble rotenone, MPTP or its metabolite MPP+ that may inhibit mitochondrial complex I and may produce the adverse outcome of symptoms as Parkinsonian disorder. Untiring efforts of the highly qualified scientists who served as the reviewers are greatly appreciated. They devoted their time in reviewing the AOP draft, identifying the deficiencies and main issues and participating in the teleconference (TC) held on May 16, 2017. During the TC, each issue was discussed and the authors provided adequate response, discussion and explanation, which are described in this report and will be reflected in the revised AOPWiki. All reviewers were satisfied with authors' responses. The authors are greatly appreciated for their input to this AOP and the external review process. Finally, I would like to thank the OECD administrators (Magdalini Sachana and Nathalie Delrue) for their assistance to the reviewers, authors and the review manager at every step of the review process.