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Adverse Outcome Pathway External Review Report

AOP 17: Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory

Short name: Oxidative stress and Developmental impairment in learning and memory

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| This document has been prepared by the review manager of AOP 17 scientific review.  It compiles the views and comments of the reviewers and explains how the authors of the AOP plan to address these comments.  It provides the basis to EAGMST for determining if AOP 17 has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and the Working Party on Hazard Assessment for endorsement. |

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# Introduction and background to specific AOP

## Background

AOP 17 ("Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory") passed internal review and was approved for release to external/scientific review at the OECD EAGMST annual meeting in June 2018. In response to requests to WNT (letters ENV/EHS/ND/2018.10 and ENV/EHS/ND/2019.01), the review manager was nominated in June 2019. Four external reviewers nominated by the review organiser completed their reviews between July and September 2019. These reviews were collated by the review manager and sent to the AOP developer on 27 September 2019. An end-of-review teleconference (TC) was held on 29 October 2019. The developer provided responses to the reviewers on 18 October 2019 and 17 March 2020.

This external review procedure is based on the "Draft Standard Operating Procedure for Adverse Outcome Pathway Scientific Review", Revised 20 November 2018.

Review organiser/manager: Magda Sachana, OECD Secretariat

## Introduction

AOP 17 ("Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory") describes the linkage between binding to sulfhydryl(SH)-/seleno-proteins involved in protection against oxidative stress and impairment in learning and memory, the Adverse Outcome (AO). Binding to SH-/ seleno-proteins involved in protection against oxidative stress has been defined as the Molecular Initiating Event (MIE). Production, binding and degradation of Reactive Oxygen Radicals (ROS) are tightly regulated, and an imbalance between production and protection may cause oxidative stress, which is common to many toxicity pathways. Oxidative stress may lead to an imbalance in glutamate neurotransmission, which is involved in learning and memory. Oxidative stress may also cause cellular injury and death. During brain development and in particular during the establishment of neuronal connections and networks, such perturbations may lead to functional impairment in learning and memory. Neuroinflammation (Resident cell activation; Increased pro-inflammatory mediators) is triggered early in cell injury cascades and is considered as an exacerbating factor. The weight-of-evidence supporting the relationship between the described key events is based mainly on developmental effects observed after an exposure to the heavy metal, mercury, known for its strong affinity to many SH-/seleno-containing proteins, but in particular to those having anti-oxidant properties, such as glutathione (GSH). The overall assessment of this AOP is considered as strong, based on the biological plausibility, the empirical support and on the essentiality of the Key Events (KEs), which are moderate to strong, since blocking, preventing or attenuating an upstream KE is mitigating the downstream KE. The gap of knowledge is mainly due to limited quantitative evaluations, impeding thus the development of predictive models.

# Synthesis of main issues of the review

Main issues from the reviewers are summarised in Annex 2, which were sent to the developer on 27 September 2019. In the same Annex, the responses to the reviews provided by the developer on 18 October 2019 are included that indicate also the updates/changes in the AOP-Wiki.

The main issues identified in the reviews that formed the main discussion points at the teleconference are described below:

## Summary of responses to CQ 1 - Scientific Quality

There was a general agreement that the AOP incorporates the most important scientific literature and current scientific knowledge in this field. Additional references were suggested for consideration.

However, concerns were raised for the limited number of publications in the Uncertainties and Inconsistencies sections given the technical challenges and the inherent variability issues associated with the assessment of neurotoxicity and neurobehaviour. **To discuss** if a general remark on this issue with reference to key papers is sufficient or if there is need to extensively list all the relevant papers.

In addition, there was interest and suggestion to add additional examples from other neurotoxicants for the different KEs leading to the AO that might not affect the same MIE (binding to SH/SeH) and not restrict the only to information that is based to very few substances (Hg, MeHg, acrylamide) that act through the specific MIE. **To discuss** if these additional empirical evidence are essential for the robustness of the AOP.

Specific comments were related to the following KEs and KERs:

* KE1538 Decreased protection This Key Event is poorly documented. There should be plenty of studies demonstrating that decreased levels of GSH and other antioxicand enzymes lead to greater sensitivity (less protection) to oxidative stress. **To discuss** renaming of the KE into decreased capacity to reduce oxidative stress and the existence of supporting evidence to be included.
* KE188 Neuroinflammation It was questioned what evidence exist that neuroinflammation occurs during neural development as only neurodegeneration is listed here under Domain of Applicability. **To discuss** the role/existence of this process in neurodevelopment and the existence of evidence to support that.
* KE1488: Glutamate dyshomeostasis There is another mechanism for L-Glu toxicity other than excitotoxicity, actually. This mechanism is called ‘oxidative glutamate toxicity’ and authors had better refer because this mechanism is directly related to the oxidative stress. Increased extracellular L-Glu lead to reverse action of Cys-L-Glu antiporter, GSH depletion. The resulting decrease in intracellular Cys influence the capacity of cells to scavenge free radicals (Kritis et al., 2015, Frontiers Cell Neurosci 9: 91). **To discuss** the inclusion of this mechanism.

## Summary of responses to CQ 2: Verify the weight of evidence judgement/scoring provided by AOP developers for KEs, KERs and the overall AOP

Reviewers generally agreed with the scoring of the weight of evidence (WoE) for the KEs and KERs. Some clarifications and additional considerations were requested for:

* KER1765: Binding, SH/SeH proteins involved in protection against oxidative stress leads to Protection against oxidative stress, decreased, related to (1) quantitative information that would strengthen the evidence that on a molar basis, biologically-relevant Hg/MeHg concentrations are sufficient to bind to and inactivate a fraction of GSH and SH/SeH antioxidant proteins large enough to significantly impair antioxidants defences and lead to increased oxidative stress in vivo, (2) the fact that Hg/MeHg can also cause neurotoxicity through numerous other molecular mechanisms that may also result in oxidative stress and the assessment of antioxidant enzymatic activities as an estimation of SH/SeH binding for the MIE may not be appropriate or conclusive, as exposure to Hg/MeHg may have overwhelmed antioxidant defences through other molecular mechanisms. **To discuss** (1) if there are quantitative evidence or examples of a drug or chemical that acts on this pathway in a highly specific manner to be included and if not how this would affect the scoring given to the KER and (2) the potential inclusion in “uncertainties” that most neurotoxic chemicals that induce oxidative stress also disrupt other cellular mechanisms and it is often unclear whether the oxidative stress is a direct or secondary response, which means that this proposed AOP likely is closely associated with other (perhaps as yet undefined) AOPs.
* KE 188 N/A, Neuroinflammation, KE 1492 Tissue resident cell activation and KE 1493 Increased Pro-inflammatory mediators related to the Neuroinflammation, Tissue resident cell activation and Increased pro-inflammatory mediators KEs which all share relatively similar endpoints. This is a major issue in the AOP framework where every KE should be distinct and measurable. **To discuss** the [outcome of a workshop](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6309953/) on how to describe inflammation as KE and how to proceed.
* KER 365 Cell injury/death leads to neuroinflammation related to the molar concentrations of many of the Mercury studies cited in the section “Emperical Evidence”, as they are in mM concentrations. Such concentrations of mercury in vivo are not relevant. Many of the studies cited for mercury and all cited for acrylamide are not developmental, thus the level of evidence for this in developing brain is minimal. Therefore, it’s advised to characterise the level of evidence for this KER in a developmental context to be LOW, not Moderate. As with other KERs, many of the sections of this KER are not completed. **To discuss** the scoring provided for this KER and if it needs to bechanged.
* KE 386 Decreased neural network function Network function can be measured at all life stages, The write up focuses specifically on development of synaptic activity. While there are clear differences in network function during development and in the adult, this write up only covers the developmental aspects. Either the name ofhte KE should be changed to reflect the developmental focus, or the description of the KE should be amended to include information about neurotransmission and network function in the adult.
* In contrast to the key event description, the section on how it is measured in vitro, especially that focusing on microelectrode arrays, is outdated and focused on acute measures in “mature” networks. Many brain regions besides cortical neurons can be recorded from using this approach (including peripheral tissue such as Dorsal Root Ganglion cultures, and the approach can be utilized with neurons from mice, rats and human iPS-derived neurons. Further, Brown et al., 2016 published a proof of concept “network formation assay” that examines effects of toxicant exposure during 12 days in vitro of network formation. This has been followed by two more studies (Frank et al., 2017 and Shafer et al., 2019) that have examined approximately 200 chemicals in this assay.
* The “Domain of Applicability” section of this KE is also inadequate. The electrophysiological approaches mentioned in the “How it is measured” section can be applied to many mammals (especially mice, rats and humans (in vitro through the use of iPS derived neurons. The comment about variability among species here, while true, also ignores evidence that there is also great conservation of the patterns of activity across species when examining the same brain regions. **To disscuss** renaming of the KE or inclusion of additional information.
* KER 359 Decreased network formation and function to Impairment in learning and memory P9, line 25 from the bottom: ‘Interestingly, behavioral alterations were detected long time after exposure (delayed effects)’. The authors had better take the distribution of MeHg into account for the phenomena described above. MeHg could readily cross the blood brain barrier via the L-type large neutral amino acid transporter and distribute to all areas of the brain (Yin et al., 2008, JNC 107, 1083-1090). MeHg then preferentially accumulates in astrocytes (and to some degree in microglia), resulting in astrocytic swelling (Charleston JS et al., Toxicol. Appl. Pharmacol. 129 1994, 196-206; Charleston JS et al., Neurotoxicology 17, 1996, 127-138; Tiffany-Castiglion E, Qian Y., Neurotoxicology. 2001 Oct;22(5):577-92.). Likewise, autometallography demonstrated that mercury was primarily located in glial cells of brain samples from autopsies (Pedersen MB et al., Int J Circumpolar Health. 1999 Apr;58(2):96-107.) These astrocyte-specific distribution is one of the reasons for taking long time to reveal behavioral effects. **To discuss** if this explanation increases the weight of evidence provided by these studies (Orenstein et al., 2014; Yorifuji et al., 2011).
* KER865: KE2 (Oxidation, Uroporphyrinogen) leads to KE3 (UROD inhibition), related to uncertainty with the identity of the UROD inhibitor and also the process/pathway in which the inhibitor is generated. KER865 was identified as the “weakest link” in this pathway by one reviewer.

## Summary of responses to CQ 3: Additional observations or comments (e.g. what do you consider to be critical data gaps and how to fill them in, how effectively the AOP communicates specialized knowledge to a non-expert audience, as simple as possible, but not simpler).

General comments:

* Trying to link binding to the SH/SeH groups of antioxidant proteins by Hg/MeHg (whose pleiotropic effects in vivo may blur the interpretation of the results) to impairments in learning and memory may be overly ambitious. Wouldn’t it be preferable to start smaller, with KE1 as the new MIE (which may read as “metal-induced decrease in protection against oxidative stress”)? This approach would help to solve the AOP structural issue identified in CQ2, avoid a few assumptions (also discussed in CQ2) and allow the use of a greater variety of stressors (as AOPs should preferably not be chemical-specific). Once a simpler, well-structured AOP relying on less assumptions and applicable to a wider range of stressors is endorsed, it will always be possible to build another AOP attempting to link inactivation of SH/SeH groups on protein involved in oxidative stress defence to impairment of learning and memory, reusing the same KEs and KERs. **To discuss** the potential renaming of KE1 and change into MIE.
* KER 1766 Protection against oxidative stress, decreased leads to Oxidative Stress. The sections on “Response-Response”, “Time-scale”, “Modulating factors” and “feedback loops” for this KER are not populated. This is a well studied phenomenon, and there should be ample literature to provide at least some information in these sections. As such, this KER is somewhat imcomplete. **To discuss** the potential of including quantitative information.
* P45, line 17: Authors should describe the protocol of water maze. **To discuss** if the protocol is needed or not.
* Despite this AOP is for DNT, the information concerning the relationship between redox state and neural stem cells and/or neural precursor cells is insufficient. For neural stem cells, redox state regulate their proliferation, maintenance, and differentiation (Iqbal and Eftekharpour, Stem cells international 2017 9209127; Wilson et al., Seminars in cell and developmental biology 80 2018, 43-9; Borfquez et al., J. Neurochem. (2016) 137, 506–517.). The most common ROS members are the superoxide anion, the hydroxyl radical, and hydrogen peroxide (Wilson et al., Seminars in cell and developmental biology 80 2018, 43-9). Growth-promoting external stimuli that can elevate intracellular ROS levels predominantly originate from plasma membrane-bound NADPH oxidase (NOX). These ROS signals result in the inhibition of protein tyrosine phosphatases (PTP) in their vicinity, through oxidation-driven structural changes. PTPs at these sites are required to modulate the receptors’ sensitivity for growth factors. This phenomenon is involved in the regulation of cell proliferation. EGF receptor is one of the well-studied signaling pathways that is involved in the regulation of NPC proliferation in developing mouse brain. The activation of EGF receptors and the enhancement of cell proliferation in this system is known to be mediated by transient increase in H2O2 levels. Low ROS levels are important for cell proliferation and limit differentiation. The rise in ROS levels or exposure to stressful conditions inhibits proliferation and promotes cell differentiation. On the other hand, several transcription factors are directly regulated by ROS-mediated oxidation of their thiol groups located on cysteine residues. These include ATM, FoxOs, Nrf2, HIF1α, and APE1 that are known to regulate redox-driven signals with regard to NPC fate determination. Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase involved in redox balance, DNA repair, and cell proliferation. The presence of PTEN results in NPCs exiting cell cycle at the G0 phase and increased self-renewal by AKT1 down regulation. Astrocyte differentiation pathway is altered but NPC proliferation is unaffected upon PINK1 knockout. **To discuss** the importance of including this additional information on redox state and neural stem cells and/or neural precursor cells.

# Summary record of the teleconference

**29 October 2019**

End-of-review teleconference (TC) was held on 29 October 2019. It was attended by all reviewers, the author and the review organiser/manager (Annex 1).

* 1. **Main issues and responses during the call**

For each issue there was a short introduction by the Review Manager (RM) followed by reviewers emphasising and/or clarifying the point of their comment. Authors’ initial responses and further discussion led to agreement on way forward to best address the particular issues and an action list was formed.

### Issues related to CQ 1 - Scientific Quality

- All reviewers were in agreement that brain is a very specific organ in terms of redox state regulation and that additional information should be added in the relevant KE and abstract of the AOP.

*Response:*

Authors agreed to include additional information in the KE oxidative stress and abstract.

- One reviewer expressed concern for the limited number of publications in the Uncertainties and Inconsistencies sections given the technical challenges and the inherent variability issues associated with the assessment of neurotoxicity and neurobehaviour.

*Response:*

Authors agreed to include a general remark on this issue with reference to key papers that will be provided by the reviewer.

- Review rose the issue that additional examples from other neurotoxicants for the different KEs leading to the AO that might not affect the same MIE (binding to SH/SeH) and not restrict the only to information that is based to very few substances (Hg, MeHg, acrylamide) that act through the specific MIE could increase the robustness of the AOP.

*Response:*

Authors recognised the value of data from additional chemicals, but they were reluctant to work on this issue because the AOP Wiki automatically links stressors to the KEs and KERs as they are added from the authors.

The RM informed the group that EAGMST has just initiated an effort to map chemicals to KEs as at the moment there is no guidance on it.

It was agreed that no action is needed.

- Reviewers have questioned if KE1 (Protection against oxidative stress, decreased) should be the actual MIE (currently named Binding, SH/SeH proteins involved in protection against oxidative stress) of the AOP.

*Response:*

Authors explained that the previous version of this AOP had merged MIE and KE1 going directly from MIE: binding to Se/SH proteins to KE oxidative stress. However, the reviewers who made the internal review disagreed with that and asked them to separate MIE and KE1. Therefore, they introduced the KE: Decreased in protection against oxidative stress. They are aware that the methods of measurement of MIE and KE1 are similar and that the binding to these proteins induced a loss of their function. They do not think that they could suppress MIE and use KE1 as MIE, because the MIE must describe the chemical interference, not the consequence of the interference.

Reviewers commented that currently MIE and KE1 are both measured by establishing antioxidant SH/SeH protein activities, which do not permit any discrimination between the MIE and KE1. In addition, very few manuscripts appear to have directly assessed SH/SeH binding, which may not be an endpoint well suited for regulatory purposes. Another issue that was highlighted by the reviewers was that the actual proteins altered by Hg, MeHg and Acr might differ.

RM reminded that the description of the chemical interference in an MIE was conceived initially as it was important for the establishment of QSARs. She pointed out that there are AOPs that use as MIEs the consequence of the interference rather than the interaction and proposed that a dialogue should initiate with the internal reviewers and reach consensus.

- One reviewer was concerned that the KE1 (Protection against oxidative stress, decreased) is poorly documented as regards the documentation of studies indicating that decreased levels of GSH and other antioxidant enzymes lead to greater sensitivity (less protection) to oxidative stress.

*Response:*

Authors agreed to include the information in the KER Protection against oxidative stress, decreased leads to Oxidative stress.

- Reviewers expressed again concern about having KEs within the AOP that are not clearly measured by distinct methods like in the case of the MIE and KE1. This time the comment was referring to the hub of KEs related to inflammation. A reviewer also questioned what evidence exist that neuroinflammation occurs during neural development as only neurodegeneration is listed here under the Domain of Applicability.

*Response:*

Authors explained that they introduced the general KEs used to describe Inflammation in all tissues as it was discussed in a [dedicated workshop](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6309953/). Nevertheless, in order to allow networking with the AOPs developed before the workshop, they introduced also the KE Neuroinflammation. They think that this compromise is especially important for the brain, where the concept of neuroinflammation is used in all publications. They agreed to add a brain section in the KE “increased, pro-inflammatory mediators”.

Reviewers expressed their preference in keeping the neuroinflammation as one KE rather than breaking it into subKEs as regulators do not usually see data on pro-inflammatory mediators. A reviewer expressed the view that the development stage of brain when neuroinflammation occurs is critical and that in the AOP the time lapse between this event and the next KE is not clear captured (Decreased Neural network formation and function) and other KEs that might occur in between, such as decreased dendritic pruning and synaptogenesis.

Authors explained that AOPs are simplified versions of biology and that by including more KEs that might are not available in the AOP Wiki, the creation of AOP networks might be compromised. However, they agreed to add effects of microglia and astrocyte on synaptogenesis and dendritic pruning in a specific sub-chapter called Neuroinflammation and brain development in the KE Neuroinflammation as there is no KER connecting Neuroinflammation and Decreased Neural network formation and function.

- For completeness, a reviewer requested the inclusion of the mechanism ‘oxidative glutamate toxicity’ in KE 1488: Glutamate dyshomeostasis, as seems more relevant than excitotoxicity.

*Response:*

Authors and the rest of reviewers agreed with the inclusion of this mechanism in the KE. Looking at that more closely, I think that it would be better to include this mechanism in the KER oxidative stress to Glutamate dyshomeostasis. In the sub-section feedback or modulating factors.

- A reviewer questioned the naming of the KE 386 Decreased neural network function because the focus of the text is on development of synaptic activity and that should be reflected in the title otherwise additional text should be added to cover differences in network function during development and in the adult. In addition, the reviewer pointed out that the section on microelectrode measurement is outdated.

*Response:*

Authors indicated that renaming of the KE is not necessary and that they could follow up on the inclusion of new content with the principle author of the KE. However, the reviewer who made the comment had to leave the call early and it was not clear if he would be satisfied with the way his comment was addressed. RM suggested to follow up on this after the call with the reviewer.

### Issues related to CQ 2 - Weight of evidence judgement/scoring

- One reviewer questioned the quantitative understanding call ‘moderate’ for KER 1765: Binding, SH/SeH proteins involved in protection against oxidative stress leads to Protection against oxidative stress, decreased. The reviewer also requested the inclusion under “uncertainties” a paragraph explaining that most neurotoxic chemicals that induce oxidative stress also disrupt other cellular mechanisms and it is often unclear whether the oxidative stress is a direct or secondary response.

*Response:*

Authors and reviewers agreed that this point is closely related with the issue discussed earlier on having KE1 (Protection against oxidative stress, decreased) as the actual MIE of the AOP rather than the existing one (Binding, SH/SeH proteins involved in protection against oxidative stress). Depending on the outcome of the discussion with the internal reviewers, appropriate action will be taken by the authors to amend the scoring or not.

Authors agreed to add in uncertainties section of the first KER a comment whether oxidative stress is a direct or a secondary response to neurotoxic chemicals.

-Reviewers questioned the evidence call ‘moderate’ for KER Oxidative stress to Glutamate dyshomeostasis.

*Response:*

Authors agreed to change the scoring from moderate to low. The reviewer also requested the inclusion under “uncertainties” a paragraph stating that the evidence used derived mostly from studies using high concentrations of mercury and acrylamide that are not relevant and not directly linked to neurodevelopmental.

- One reviewer suggested the inclusion of information on astrocyte-specific distribution of mercury to explain the late neurobehavioral effects in KER 359 Decreased network formation and function to Impairment in learning and memory.

*Response:*

Authors initially agreed to include the information in the uncertainty section of the KER. After some discussion, it became clear that this information do not really explain the delayed effects. Agreed that authors will include the information where they found that is most suitable.

### Issues related to CQ 3 - Additional observations or comments

#### General comments:

- One reviewer pointed out that the quantitative understanding of the KER 1766 Protection against oxidative stress, decreased leads to Oxidative Stress is very well studied. However, limited information is included in this section.

*Response:*

RM explained the information that needs to be captured under the quantitative understanding section. Authors indicated that some quantitative information like response-response data and time scale is provided in the table of the empirical support. Reviewer seemed satisfied and no further action is needed.

- One reviewer requested the description of the protocol on water maze.

*Response:*

All agreed that a citation should be enough.

- One reviewer pointed out that the information concerning the relationship between redox state and neural stem cells and/or neural precursor cells is insufficient.

*Response:*

Authors agreed to include information on the non-adjacent KER linking oxidative stress to cell injury/death, making a specific chapter on effects during brain development.

#### Specific Comments:

Limited time was left to discuss the specific comments. Only the concern about the table on L-Glu transporters was discussed and agreed that the reviewer will look at it again and come up with further suggestions. Authors explained that the table is from a published paper by a scientist with expertise in the field.

### Action list

1. Include additional information that shows that brain is a very specific organ in terms of redox state in the relevant KE and abstract.

* reviewers to provide specific references to be considered

ACTION: A small paragraph on the physiological features making the brain more susceptible than other organs to oxidative stress has been added in KE oxidative stress.

1. Include brief comments on technical challenges and the inherent variability issues associated with the assessment of neurotoxicity and neurobehaviour in Uncertainties and Inconsistencies sections in Appendix 2 on page 9.

* reviewers to provide specific references to be considered. After the call, one of the reviewers sent a reference to be included and to address the uncertainty surrounding the assessment of mercury’s effects on neurodevelopment in different human populations. Grandjean, P. (1999). Mercury risks: controversy or just uncertainty? Public Health Reports, 114(6), 512.

ACTION: A discussion about uncertainties and discrepancies surrounding mercury’s effects on neurodevelopment in different human populations has been added in the overall assessment of the AOP.

1. RM to contact the internal reviewers and explore if consensus can be reached on having KE1 as MIE.

ACTION: Specific methods to measure binding of mercury and acrylamide to Se/SH proteins involved in protection against oxidative stress have been added in MIE (see specific comments). With that, MIE and KE1 have their own specific methods and the reviewers agreed that this action was sufficient to keep MIE and KE1 as proposed here after the corrections of the internal review.

1. Include additional information in the KER Protection against oxidative stress, decreased leads to Oxidative stress to better document that decreased levels of GSH and other antioxidant enzymes lead to greater sensitivity (less protection) to oxidative stress.

ACTION: We think that the description of this KER1766 is quite straightforward: when protection against oxidative stress decreases, there is an increase in oxidative stress. We can’t think of anything else to add.

1. RM to contact some authors of the workshop report and investigate the possibility to keep only neuroinflammation as KE.

ACTION: A text has been added in Background section at the beginning of the AOP to explain why we finally decided to keep “Neuroinflammation” as a KE and not replace it by the two KEs “Tissue Resident cell activation, and Pro-inflammatory-mediators, increased” as proposed in a workshop dedicated to Inflammation. The two main reasons to keep neuroinflammation as a KE are that Neuroinflammation is a concept recognized by the regulators and is found in the whole literature describing brain inflammation.

1. Include a developmental brain section in the KE “Neuroinflammation” and information related to synaptogenesis and dendritic pruning.

ACTION: a section on Neuroinflammation and Brain Development has been added.

1. Include the mechanism ‘oxidative glutamate toxicity’ in KER: Oxidative stress to Glutamate dyshomeostasis.

ACTION: The mechanism “oxidative glutamate toxicity\* has been added in the KER oxidative stress leads to Glutamate dyshomeostasis in the subchapter “Feed forward/Feedbackloops”

1. RM to contact the reviewer regarding the potential renaming of the KE 386 Decreased neural network function or the inclusion of text in differences in network function during development and in the adult.
2. Include additional text on microelectrode measurements in KE 386 Decreased neural network function as indicated by the reviewer by liaising with the principle author of the KE.
3. Await for the outcome of the discussion with the internal reviewers and then change the scoring if necessary of the KER 1765: Binding, SH/SeH proteins involved in protection against oxidative stress leads to Protection against oxidative stress, decreased. Once resolved add in the uncertainty section of the first KER text related to whether oxidative stress is a direct or a secondary response of neurotoxicants.

ACTION: The scoring of the KER has been rated MODERATE, since there are other Se/SH proteins , i.e. not involved in protection against oxidative stress, where the chemical initiators could bind, and as assessed in several uncertainties sections, because oxidative stress could be a primary or a secondary response of neurotoxicants.

1. Change the scoring from moderate to low for KER Oxidative stress to Glutamate dyshomeostasis

ACTION: The scoring was modified as suggested by the reviewers.

1. Include information on astrocyte-specific distribution of mercury in the most appropriate KER.

ACTION: We did not include this information because it is not related to the KEs of this AOP.

1. Add citation for water maze.

ACTION: Two references have been added (Morris, 1981; Vorhees and Williams, 2006).

1. Include information related to neural stem cells and/or neural precursor cells in KER 1690 linking oxidative stress to cell injury/death.

ACTION: The following sentences have been added in KER1690: “However, it has to be noted that neural stem cells distinguish themeselves from post-mitotic neural cells by their lower ROS levels and higher expression of the key antioxidant enzymes glutathione peroxidase. This increased "vigilance" of antioxidant mechanisms might represent an innate characteristic of NSCs, which not only defines their cell fate, but also helps them to encounter oxidative stress (Madhavan et al., 2006).”

1. Reviewer to check the table on L-Glu transporters and report back.

ACTION: New section about methods has been provided by the reviewer and incorporated in AOP-wiki.

# Outcome of the external review

Developers have implemented all agreed changes and provided responses to the reviewers (18 October 2019 and 17 March 2020); the AOP-Wiki has been correspondingly updated (17 March 2020).

AOP 17 is now (17 March 2020) considered acceptable by the reviewers.

**Annex 1: List of Reviewers, Author and Review manager**

|  |  |  |
| --- | --- | --- |
| Expert name | Affiliation | Representing country |
|  |  |  |
| Guillaume Pelletier | Health Canada | Canada |
|  |  |  |
| April Kluever | Office of Management and Budget | US |
|  |  |  |
| Kaoru Sato | Centre for Biological Safety and Research  National Institute of Health Sciences | Japan |
|  |  |  |
| Tim Shafer | US EPA | US |
|  |  |  |

|  |  |
| --- | --- |
| Author | Affiliation |
| Florianne Tschudi-Monnet | Department of Physiology  University of Lausanne  Switzerland |
| Marie-Gabrielle Zurich-Fontanellaz | Department of Physiology  University of Lausanne  Switzerland |

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| Review Manager | Affiliation |
| Magda Sachana | OECD Secretariat |

**Annex 2 Main issues from the reviewers and responses**

p.3. Regarding the claim that hippocampus is particularly sensitive to the neurotoxicity of mercury: The Morris et al., 2017 reference is a speculative manuscript on a potential link between Hg effects on the immune system where I could not find anything on the comparative sensitivity of the hippocampus. Similarly the Sokolowski et al., 2011 reference claims to report cell death in the hippocampus at lower MeHg level than previously reported, but do not directly compare hippocampus sensitivity to other brain structures. I could not find a direct comparison in the Ceccatelli et al., 2013 reference either. A quick search identified a manuscript showing higher sensitivity to MeHg-induced oxidative stress in the cerebellum compared to the hippocampus (PMID:1475065) and a review providing a few other references on MeHg selective toxicity towards the cerebellum (<https://doi.org/10.1016/j.toxlet.2013.08.002>). Hence, endpoints related to perturbation of cerebellar functions may also make very appropriate and biologically plausible AO.

RESPONSE: We will modify according to the reviewer’s comment.

ACTION: We removed the adverb “particularly” before “susceptible”, in order not to mention that hippocampus is more susceptible to mercury toxicity than other brain areas. The chosen AO of this AOP is “impairment of learning and memory”, therefore we concentrate on the areas involved in the process. The goal is not to compare the susceptibility of the different brain areas.

p.4. The last reference of the paragraph (Vather et al., 2006) also presents a few human epidemiology data on sex-specific effects of mercury exposure that may be worth further discussion.

RESPONSE: Again this is the Overall Assessment. We could include more informations for that in the related KER if the reviewer clarifies his/her request.

ACTION: The discussion of some sex-specific effects has been done in the AOP and the paper of Vahter et al, 2006 suggested some explanations of these differences. It seems to us that this level of precision is sufficient for the OA.

p.4. For the essentiality of MIE, it may be appropriate to clearly mention that the decreased protection against oxidative stress may not only be due to the inactivation of SH/SeH groups on protein involved in defence against oxidative stress, but also to other Hg/MeHg mechanisms of toxicity. Is the Reeves 2009 citation in fact the Reeves and Hoffmann, 2009 publication listed in the References section?

RESPONSE: Yes it is the reference of Reeves and Hoffmann (2009). This will be corrected.

ACTION: As we removed MIE from the table of essentiality, see below, the correction is not necessary.

Page 4 table: KE1 Rationale: I recommend striking out “since it is part of the definition of socidative stress” since the definition is irrelevant here.

RESPONSE: It will be removed as suggested by the reviewer.

ACTION: done

Page 4 table: How can the MIE have only “moderate” essentiality? By definition, isn’t the MIE essential to the entire AOP?

RESPONSE: Reviewer is right. MIE should not appear in the table of essentiality of the KEs.

ACTION: MIE has been removed from this table.

p.4. For the essentiality of the KE3, it may be a good idea to introduce the term excitotoxicity.

RESPONSE: Excitotoxicity is already mentioned in the descriptin of this KE. In the table of essentiality of the KEs, since it is only a summary we don’t want to emphasise excitotoxicity over the role of glutamate as trophic factor.

NO ACTION REQUIRED

Page 5: “pre-natal exposure to methylmercury.” – Is this in rats? Please specify

RESPONSE: It is school-aged children (cf reference). This information will be added.

ACTION: done

p.5, AO. Given that this AO is the main concern of regulators, it may be worth presenting and discussing human epidemiological results at high versus low doses and the discrepancies between Seychelles and Faroe Island studies.

RESPONSE: We will include these results. If the reviewer could provide the specific references, it would be a great help. Thanks.

ACTION: The discussion about the discrepancies in epidemiological studies has been introduced in the text of the OA .

WoE, MIE, p.6. In the Ruszkiewicz 2016 publication, it is the activity of the proteins that was measured and not the binding to SH/SeH, this endpoint clearly belongs to KE1 and not MIE. The same issue is present with many of the publications listed in this table. According to OECD guidance documents the MIE is a specialized KE and for every KE there should be a measurable endpoint. If the authors want to properly document this MIE they should present the Trx and Grx dimers, the DTNB titration of thiol groups and the mass spectroscopy data following exposure to HgCl2 described by Carvalho 2008 (doi: 10.1074/jbc.M710133200Is). The Carvalho 2008 manuscript also present data on the the molecular ratio of mercuric compounds required to disrupt thiol groups on Trx, an important topic, as stoichiometric considerations are important to establish biological plausibility of the KER between MIE and KE1. If the authors want to properly defend this MIE they will need to find and present additional studies that directly focus on SH/SeH binding. As previously mentioned Hg/MeHg may cause decrease in intracellular defence against oxidative stress through other molecular mechanisms. Starting the pathway with a MIE named “Metal-induced decrease in protection against oxidative stress” would eliminate uncertainty about the other mechanisms by which Hg/MeHg may induce oxidative stress and allow to use a more diverse array of inducer such as lead, cadmium, arsenic, etc… Regarding the Wagner et al., 2010 publication, it is worth noting that although MeHg inhibited thioredoxin in a brain protein extract, in vivo exposure of mice to up to 10 mg/kg MeHg did not affect thioredoxin activity in brain. Hence this study should be cited somewhere in the Uncertainties and Inconsistencies sections of Appendix 2.

RESPONSE: See responses to main points in general comments. The reference of Carvahlo et al (2008) will be added. We will add also the paper of Wagner et al., 2010 in Uncertainties of the KER as suggested by the reviewer.

ACTION: A new chapter about methods measuring the binding of toxicants to the SH/Se/H groups of proteins has been added in MIE.

WoE, KE3, p.6. The Juarez 2002 citation is not listed in the reference list. I am assuming that numerous chemicals can affect glutamate homeostasis. Listing a few of them may help to increase this WoE.

RESPONSE: Reference of Juarez et al., 2002 will be added in reference list.

Sure that other chemicals may affect glutamate homeostasis, therefore, this KE “Glutamate Dyshomeostasis” could be re-used by other AOPs in development. But in this AOP17, we can only consider the specific chemical stressors of this specific MIE.

ACTION: Reference has been added.

WoE, AO, p.7. Given that the impairment of learning and memory in human will be the main point of interest of regulatory toxicologists, the human subsection of the table should further develop the AO in human, by presenting the neurological effects of Hg/MeHg at high dose (Minamata, Irak) along with the main epidemiological studies at lower doses (Seychelles, Faroe Island).

RESPONSES: These references will be added as suggested by the reviewer.

ACTION: These epidemiological studies have been added in the AO in the table entitled “Dose response and temporal concordance. And the discussion about some uncertainties raised by divergent results of these epidemiological studies has been included in the text of the OA.

Biological plausibility of KER, p.7. General comment on this table: A lot of the examples provided are for Hg/MeHg. Providing examples from other pollutants or pharmaceutical may be appropriate, as these KERs should be designed to be easily reusable in other AOPs.

RESPONSE: This table summarizes the main points of AOP17. Informations about other chemicals would be found in the empirical support of the KERs, if KERs are shared by another AOP (AOP networking).

NO ACTION REQUIRED

Biological plausibility of KER, MIE to KE1, p.8. The authors clearly demonstrated that Hg /MeHg can bind to and inactivate SH/SeH proteins. However, GSH is present in high concentrations (1-10 mM according to you description of KE 1538 on p.22) and there are multiple other SH/SeH target beside proteins involved in the regulation of oxidative stress. Given the very limited number of stressor associated with this AOP (Hg and MeHg) that may also induce oxidative stress through other molecular mechanisms of toxicity, unless you can present data suggesting that biologically-relevant brain Hg/MeHg concentrations are sufficient to inhibit a significant fraction of the proteins involved in response to oxidative stress, the biological plausibility of the MIE cannot be higher than moderate.

RESPONSE: We agree with the reviewer that the chemical initiators could bind to other Se/SH. But according to the internal review we had to restrict the MIE to the proteins involved in protection against oxidative stress, which are inactivated by the binding we rated this KER as HIGH. We are not aware of a reference suggesting that biologically-relevant brain Hg/MeHg concentrations are sufficient to inhibit a significant fraction of the proteins involved in response to oxidative stress, therefore we can change the biological plausibility from gigh to moderate.

ACTION: The score for this KER has been changed from HIGH to MODERATE, and a sentence has been added: However, binding to other SH/SeH groups of proteins not involved in protection against oxidative stress can occur and trigger other neurotoxicity pathways.

AOP 17, Biological plausibility of KER, KE3 to KE4, p.8. The Boron and Boulpaep 2003 citation is missing from the References section. As the authors admit themselves, there is a serious causality issue here. If they want to rate this KER as more than WEAK they will need to provide references that convincingly demonstrate that oxidative stress in absence of perturbation of mitochondrial function and energy metabolism is sufficient to affect glutamate homeostasis. Alternatively, the glutamate dyshomeostasis may be omitted from this AOP, and may be used as an MIE in another AOP.

RESPONSE: The reference of Boron and Boulpaep (2003) will be added in the reference list. We agree with the reviewer’s proposal and will rank the KER linking oxidative stress to glutamate dyshomeostasis WEAK instead of MODERATE.

ACTION: KER has been rated LOW as suggested and the reference has been added.

AOP 17, Biological plausibility of KER, KE4 to KE5, p.8. There is no citation for this KER. Also, AOPs are chemical agnostic and every component must stand on its own in order to be reused in other pathway. Consequently, please delete “all three chemical initiators (mercury, acrylamide and acrolein)”. I am also assuming that several other pharmaceuticals can block glutamate transport and lead to cell death.

RESPONSE: In the Overall Assessment this table is a only summary of the KER. The references are found in the description of the KER.

KEs are chemical agnostic, whereas in KERs, the chemical initiators are required in the section called empirical support.

NO ACTION REQUIRED

P8, line 16: ‘the tight coupling of glutamate transporters with energy production’

L-Glu transport itself is driven by the Na+ ion gradient and does not need ATP. Does this mean the co-localization of L-Glu transporters and Na+-K+ ATPase or a range of events following L-Glu uptake? This expression will lead readers to misunderstanding.

RESPONSE: L-GLU transport is driven by the Na+ ion gradient, which is dependent of the Na/K ATPase, which in turn requires energy. We will modify the sentence to prevent misunderstanding.

ACTION: The sentence was modified as following: Glutamate transport is driven by the Na+ ion gradient, which is dependent on the Na/K ATPase, which, in turn, requires energy. Glutamate enters the cells accompanied by 2 Na+and an H+.  Perturbations of energy metabolism such as mitochondrial dysfunction and increased production of ROS will lead to glutamate dyshomeostasis, due to the indirect coupling of glutamate transporters with ATP level, and to the important role of glutamate transporters in glutamate homeostasis.

AOP 17, Biological plausibility of KER, KE5 to KE6, p.8. Please delete “all three chemical initiators (mercury, acrylamide and acrolein)”.

RESPONSE: KEs are chemical agnostic, whereas in KERs, the chemical initiators are required in the section called empirical support.

NO ACTION REQUIRED

P9, line 11 from the bottom:

In the paper by Sandstrom, there is no description about GSH. Please check.

RESPONSE: Sorry we put the wrong reference. It is Sandström et al 2017, which includes effects of toxicants on GSH synthesis. This will be corrected.

ACTION: correction done.

AOP 17, Quantitative Consideration, p.10. Regarding the second paragraph it is important to remember that AOPs are chemical-agnostic, so there is no requirement to rely exclusively on Hg/MeHg. Please also delete the extra copy of the second paragraph and correct the Fritsche et al., citation (2017 in the text and 2017b in the References section).

RESPONSE: KE are chemical agnostic, but KER contains always empirical support with the chemical initiators.

The reference of Fritsche et al 2017 will be corrected.

The duplicated paragraph will be removed.

ACTION: Reference corrected and duplicated paragraph removed.

AOP 17, References, p.11-14. Please delete the publications not cited in the text (Fombonne, Gilbert, Hallmayer, Jafari, Li (2017 and 2018), Mostafa, Saghazadeh). Please also add the Costa et al., 2004 publication cited in the first paragraph of the Overall Assessment of the AOP, and use 2008a and 2008b for the two Castoldi et al., 2008 manuscripts.

RESPONSE: The reference list will be corrected as suggested by the reviewer

ACTION: non cited references have been removed; reference of Costa 2004 has been introduced and Castoldi et al references have been corrected.

Appendix 1, MIE 1487, p.15. In the second paragraph you mention that the reaction constants are so high that binding will occur almost instantaneously with the first SH or SeH encountered, and that consequently the reaction is controlled by diffusion. In the next paragraph you then mention that selenoproteins should be a preferential target. The constants for MeHg (Table 1) overlap, while the constants for Hg2+ (table 2) are so high that they will be uniquely controlled by diffusion. You may want to check this assumption with a chemist. There are two references for Branco 2012 and the Della Corte 2013, Meinerz 2017 and Oliveira 2017 citations are missing from the References section. Regarding the last paragraph on Hg toxicity at low doses, please see my previous comments on the ratio of Hg/MeHg to potential binding target.

RESPONSE: Firstly, it is important to clarify that a fast reaction does not mean necessarily a reaction with high or strong value of constant of formation (though it is common, there is the possibility of existence of a complex with a strong constant of formation, but formed by a reaction that occurs slowly). In the case of reactions of –SH or –SeH with CH3Hg+ or Hg2+, the values of both constant of formation and reaction rates are high. But the interaction of Hg2+ with thiol or selenol groups is faster and much more stable than with CH3Hg+. However, despite of the strong stability of the complexes Hg-S or Hg-Se, in the presence of free thiol or selenol groups, the complexes can undergo fast exchange reaction (Rabenstein’s reaction). In the text, the values of about 108 M-1.sec-1 is for this type of exchange reaction. The point here is that even after binding to a HMM-SH or HMM-SeH molecule, the mercurial (Hg2+ or CH3Hg+) can exchange with a free thiol group either from GSH, cysteine or other thiol- or selenol-containing proteins. The major limitation that we have here is the introduction of a complex thermodynamic (entropic) term when proteins (HMM-SH or HMM-SeH) are considered. The bind of MeHg or Hg2+ to thiol- or selenol-groups of proteins can cause an unfolding or a re-folding in the protein structure in such way that the targeted thiol- or selenol- group will become not accessible to the exchange reaction. However, we still have no reliable data about such effects of mercurials (for review see Nogara et al. 2019).

In relation to the question of the overlapping of constants for MeHg (Table 1) for selenol or thiol containing molecules we have to clarify that for the same thiol- or selenol-containing molecule, the constant is always high for the selenol than thiol. However, only a very small number of studies have been performed and always with low molecular mass thiol or selenol compounds. This means that we do not have the values of constant formation for the interaction between thiol-containing proteins and CH3Hg+ or Hg2+ (although there are some rate of reactions for mercurials and some thiols containing proteins). For the case of selenoproteins, there are no data available. And as commented above, the binding of mercurial compounds to proteins can cause entropic changes that can modify the exchangeability of MeHg or Hg2+ bound to the protein. Perhaps this should be incorporated in inconcistencies or gaps in our knowledge.

Regarding the point about the stoichiometry (the ratio mercurial-to-protein –SH or -SeH groups), though it is relevant from the chemical point of view, it has less significance from the point of view ot toxicology. The reasons for this is that under low toxicological levels of mercury, the concentrations of MeHg are expected to be in the nmol/L range, whereas the concentrations of the target proteins are expected to be lower than this. Indeed, the concentrations of selonol-containing proteins is very low (excepting to selenoprotein P). But unfortunately, we do not have data about the actual concentrations of the MIEs of MeHg+ and Hg2+. From the chemical constants and the behaviour of mercurial-S- or mercurial-Se- complexes we can predict with certainty that Hg2+ and CH3Hg+ will bind to –SH and –SeH within the biological fluids of human body, but we still do not know which of them have the strongest affinity for CH3Hg+ and Hg2+.

ACTION: The description of the MIE is very detailed and long, therefore we don’t want to add more complexity. Missing references have been added.

Appendix 1, MIE 1487, p.16. The Liem-Nguyem et al., 2017 and Dorea et al., 2013 citations are missing from the References section, the year is missing on the Rabestein and bravo reference. It may be a good idea to introduce Figure 2 in the main text and to better explain how exchange of MeHg from one cysteine to another cysteine or selenol group relate to the reaction constant previously provided in Tables 1 and 2. Regarding Table 3, as previously pointed out the inhibition of SH/SeH proteins protecting against oxidative stress actually belongs to KE1. Moreover it does not necessarily indicate inhibition of the protein due to mercury binding to SH/SeH, as mercury may have overwhelmed antioxidant protection through other mechanisms. Many citations from Table 3 are missing from the References section. Please specify 2012a or 2012b for the Branco et al., 2012 citation. The citation is missing for the penultimate item listed in Table 3.

RESPONSES:

Appendix 1, MIE 1487, p.16. The Liem-Nguyem et al., 2017 and Dorea et al., 2013 citations are missing from the References section, the year is missing on the Rabestein and bravo reference.

Liem-Nguyen V, Skyllberg U, Nam K, Björn E. Thermodynamic stability of mercury(II) complexes formed with environmentally relevant low-molecular-mass thiols studied by competing ligand exchange and density functional theory. Environ Chem 14:243-253, 2017.

Dórea JG, Farina M, Rocha JBT. Toxicity of ethylmercury (and Thimerosal): A comparison with methylmercury. J Appl Toxicol 33:700-711, 2013.

Rabenstein, D.L., Bravo, J. (1987)

ACTION: References have been added and corrected.

It may be a good idea to introduce Figure 2 in the main text and to better explain how exchange of MeHg from one cysteine to another cysteine or selenol group relate to the reaction constant previously provided in Tables 1 and 2.

As briefly exposed above, we have no data about the exchange reaction of MeHg or Hg2+ from cysteine or GSH (MeHg-Cys or MeHg-SG monocoordinate complexes or from Cys-Hg-Cys or GS-Hg-SG bicoordinate complexes) to target proteins (the actual MIEs) or even to abundant proteins that are important as “distributors” of mercurial but not targets of MeHg or Hg+2 (for instance, haemoglobin or albumin, for review and discussion about these issues see Nogara et al. 2019).

Regarding Table 3, as previously pointed out the inhibition of SH/SeH proteins protecting against oxidative stress actually belongs to KE1.

The question raised by the reviewer is correct. We cannot state with absolute certainity that the inhibition of –SH or –SeH antioxidant enzymes are the first targets of mercurial compounds. They can be secondary to an indirect modulation of the synthesis and/or degradation of such proteins.

Moreover it does not necessarily indicate inhibition of the protein due to mercury binding to SH/SeH, as mercury may have overwhelmed antioxidant protection through other mechanisms.

This(these) statement(s) made here is not correct. First, if MeHg or Hg2+ bind to a redox sensitive or an enzyme involved in the metabolism of pro-oxidants (for instance, GPx or TrxR) they are expected to disrupt the enzyme functioning. Thus, in the case of such enzymes that have critical selenol or thiol groups in the active site, the bind of mercurial compounds to them can trigger their inhibiton. The point here is that the mercurials can affect several different proteins by the bind to thiol- or selenol groups, but we do not know which of them are the most critical to trigger their toxicity. Indeed, it is possible and much more plausible to suppose that they will act simultaneously in several types of –SH and in some –SeH containing proteins. I realize that we have discussed these points before. I do not know if you have to state that although the MIE of MeHg and Hg2+ “have to be” -SH and –SeH groups of redox active/sensitive proteins, we still have not performed analytical and methodological approaches that have allowed us to solve these issues.

Many citations from Table 3 are missing from the References section.

Cheng JP, Yang YC, Hu WX, Yang L, Wang WH, Jia JP, Lin XY(2005) Effect of methylmercury on some neurotransmitters and oxidative damage of rats. J Environ Sci (China) 17:469-473.

Dalla Corte CL, Wagner C, Sudati JH, Comparsi B, Leite GO, Busanello A, Soares FAA, Aschner M, Rocha JBT. Effects of diphenyl diselenide on methylmercury toxicity in rats. BioMed Res Int 983821, 2013. –**In TABLE 3 it appears as 2012.**

Glaser V, Leipnitz G, Straliotto MR, Oliveira J, dos Santos VV, Wannmacher CMD, de Bem AF, Rocha JBT, Farina M, Latini A. Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury. NeuroToxicology 31:454-460, 2010b.

Glaser V, Moritz B, Schmitz A, Dafré AL, Nazari EM, Müller YM, Feksa L, Straliottoa MR, de Bem AF, Farina M, Rocha JBT. Protective effects of diphenyl diselenide in a mouse model of brain toxicity. Chem-Biol Interac 206:18-26, 2013.

Li, Y., Shi, W., Li, Y., Zhou, Y., Hu, X., Song, C., Ma, H., Wang, C., Li, Y. Neuroprotective effects of chlorogenic acid against apoptosis of PC12 cells induced by methylmercury (2008) Environmental Toxicology and Pharmacology, 26 (1), pp. 13-21.al. 1990 neuroblastoma gpx

Malagutti, K.S., da Silva, A.P., Braga, H.C., Mitozo, P.A., Soares dos Santos, A.R., Dafre, A.L., de Bem, A.F., Farina, M. 17β-estradiol decreases methylmercury-induced neurotoxicity in male mice (2009) Environmental Toxicology and Pharmacology, 27 (2), pp. 293-297.

Mori N, Yasutake A, Hirayama K. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. Archives of toxicology. 2007 Nov 1;81(11):769-76.

ACTION: All references have been added and correction in table 3 has been made.

Please specify 2012a or 2012b for the Branco et al., 2012 citation.

The citations a and b can be included after Branco et al. 2012 (i.e. it should be Branco et al. 2012 a,b)

ACTION: done.

The citation is missing for the penultimate item listed in Table 3.

This entry is about the item that is below – it has no citation.

ACTION: In fact it is the title of the columns. It put them in bold to prevent misunderstanding.

Appendix 1, MIE 1487, Acrylamide, p.16-17. All the publications cited in this small paragraph are missing from the References section. Please delete the mention to Table x (or add it to the document). As for mercury, are acrylamide concentrations observed in vivo sufficient to interfere with a significant proportion of protein involved in defence against oxidative stress?

RESPONSE: I have not found any study describing the concentration of acrylamide after in vivo exposure.

ACTION: Table x was removed.

The interpretation of acrylamide toxicity may also be blurred by other molecular mechanisms of toxicity such as impairment of membrane fusion processes leading to nerve terminal damage in peripheral nerve (LoPachin, 2005, Chemistry and Safety of Acrylamide in Food p. 21-37).

RESPONSE: The point raised by the reviewer is correct. Here, in contrast to mercurial, the constant of acrylamide for thiols is weak; consequently, it is possible that acrylamide “starts” its toxicity via disruption of other targets that via a thiol containing protein.

ACTION: No action required because the following sentence: “No literature supporting the link “SH/SeH binding leads to decreased protection against oxidative stress” for ***acrylamide as stressor*** in brain/neural tissue can be found”, was already in the uncertainties and inconsistencies section of the KER1765.

Page 17: The word “proteins” is repeated, delete one instance. Table “x” needs to be corrected to direct reader to the table.

RESPONSE: This will be corrected

ACTION: Done. Table x has been removed as constants relevant for this AOP are given in the text.

Appendix 1, MIE 1487, Key Event Description, p.18. In the sentence “Binding of chemicals to these proteins induces either their inactivation or favor their degradation and/or inhibition of their synthesis” the “inhibition of their synthesis” may requires further explanation as it is clearly not as direct as the first two mechanisms.

RESPONSE: The point here is that MeHg and Hg2+ could also (in addition to bind –SH or –SeH groups of redox active or sensitive proteins) disrupt thiol containing proteins involved in the synthesis of these redox active or sensitive thiol- or selenol-containing enzymes. The statement is very simple and perhaps, if you really realize that they are difficult to undertand, I can do a figure or a scheme.

ACTION: We removed the part on inhibition of synthesis. That was a mistake.

Appendix 1, MIE 1487, How it is Measured or Detected, p.18. As previously mentioned, the measurement of the enzymatic activities of proteins involved in protection against oxidative stress clearly belong to KE1. While measurement of enzymatic activities as an indication of inactivation of SH/SeH groups by Hg/MeHg may be appropriate for purified protein extract in vitro, there is simply too many other mechanisms by which Hg and MeHg may affect enzymatic activities beside direct interaction with SH/SeH groups.

RESPONSE: The reviewer is partially correct. In the case of an in vivo exposure, the observation of an thiol- or selenol-containing enzyme inhibition by MeHg or Hg+2 do not imply a direct interaction with thiol or selenol groups. But the inhibition in vitro can give support to this type of interaction and can suggest that such type of interaction can also occur after in vivo exposure. Although mercurial can indirectly interfere with a thiol- or selenol-containing enzyme activity, the “protein responsible for the indirect modulation” has to be a thiol- or selenol-containing protein. In short, although the primary outcome (here the inhibition of thiol- or selenol-containing antioxidant enzymes may be indirect), the modulator or the up-stream target of such indirect effect has obligatorily to be a thiol- or selenol-protein.

The analytical procedures to be used here to detected the MIE(s) should include detailed temporal proteomic analyses followed by mass spectroscopy. However, there are no such studies. Indeed, the work of Carvalho et al. 2011 performed some analyses to detect the binding of MeHg or Hg2+ to thioredoxin and thioredoxin reductase in vitro. However, the study used purified proteins and cannot be extrapolated to complex in vivo situations. In short, we have some limitations regarding the quantification and identification of the actual –SH and SeH groups that are the MIEs of mercurials.

ACTION: The citation of Carvahlo et al. 2011 for the detection of binding of mercury to thioreoxin and thioredoxin reductase has been added in the section “How is it measured”

Page 18: “Thiol (SH) – and seleno-containing proteins involved in protection against oxidative stress are mainly located in mitochondria and in the cytoplasm of the different neural cell types.” – These proteins are ubiquitously expressed across cells of all lineages – not just neural cells. As written, this sentence may incorrectly imply they may only be found in neural cells.

RESPONSE: It is included in the paragraph just above the these proteins are ubiquitous in all cell types. To prevent misunderstanding, we will add In the brain, at the beginning of the paragraph, going from general to what is brain specific.

ACTION: Done

Appendix 1, MIE 1487, References, p.19. The Rabenstein and Evans 1978, Smith, 2010 and Xu et al., 2012, 2016 references are not cited in the main text.

RESPONSE: The reference of Rabenstein and Evans can be deleted.

The reference of Smith has to be deleted.

Xu et al. 2012 and 2016 are related to the taxonomic applicability.

ACTION: Corrections have been done in the text and in the reference list.

Appendix 1, KE 1538, How it is Measured or Detected, p.22. Please note how similar the endpoints are to those listed for MIE 1487 on p.18-19. This should not happen; each KE should have its own measurable endpoints.

RESPONSE: We agree with the reviewer, but see general comments about the reason of separating MIE and KE1.

ACTION: a new chapter with specific methods measuring binding has been added.

Page 22: “… GSH serves as a reducing agent for ROS and other unstable molecules in the reaction catalysed by GPx” – GSH scavenges ROS and other unstable molecules generated by a number of catalytic systems, not just GPx. This sentence should not restrict GSH to just scavenging from GPx.

RESPONSE: We will modify this sentence according to reviewer’s suggestion (if the reviewer could provide some references it will help).

ACTION: The sentence has been modified: In fact, GSH serves as a reducing agent for ROS and other unstable molecules generated by catalytic systems, including glutathione peroxidase (GPx)(Forman, 2009).

Appendix 1, KE 1392, p.23. This KE shared with other AOP only list liver in Organ term. Is it possible to add brain and few relevant references in the event description, notably for Keap1 and Nrf2? The Jackson et al., 2014 citation in the How it is Measured or Detected section is also missing from the References section.

RESPONSE: Brain will be added in Organ Term. And we will add a small chapter on the brain specificities in the description of the KE Oxidative stress, which is a re-used KE from AOPwiki. The missing reference will be added.

ACTION: Sorry, we can’t add “brain” in the organ term of the KE1392. Please Magda, could you solve this IT problem?

A small paragraph on the physiological features making the brain more susceptible than other organs to oxidative stress has been added.

Jackson reference has been added.

ACTION 2: Liver is already removed from Organ term.

Page 23: ROS can be measured using chemiluminescence in the lucigenin/luminol system. Here is one example publication. This method could be added as a way to measure ROS, even when ROS formation is fleeting. https://www.sciencedirect.com/science/article/abs/pii/S0165993606001683

RESPONSE: This method will be added as suggested by the reviewer.

ACTION: Method added

Page 24: Under key event description, the phenomenon of excitotoxicity is described (excess glutamate resulting in cytotoxicity), but the word itself is not used. Please revise this paragraph to include “excitotoxicity” since that is the scientific term used in toxicology to express this phenomenon.

RESPONSE: The term “excitoxicity is used several times in the second paragraph of the KE description.

NO ACTION REQUIRED

Page 24: Why is there so much emphasis on astroyctes? One or two sentences should be added to explain why the Key Event focuses so much on astrocytes (as opposed to neurons, which are mentioned in the first paragraph).

RESPONSE: A sentence explained that glutamate dyshomeostasis is a consequence of perturbations of astrocyte/neuron interactions. Astrocytes play a major role in the re-capture of glutamate, also in the glutamate synthesis. Therefore it is absolutely justified to put emphasis on this cell type.

NO ACTION REQUIRED

Appendix 1, KE 1488, p.24. In the Domain of Applicability, please correct Fagnon to Fagnou in the Fagnon and Tuchek, 1995 citation. In the Key Event Description please double-check the Chai et al., 2013 citation as the title of this reference do not appear to match the context. Instead of “Genetic variants associated with autism spectrum disorders were enriched in glutamatergic pathways” it may be better to say “Genes involved in glutamatergic pathways were enriched in Genetic variants associated with autism spectrum disorders”. It may also be more appropriate to simply drop the reference to autism, which may just add to the confusion, since this AOP is about learning and memory impairments. Also, add a few words about dysregulation of intracellular calcium, which if I remember well play an important role in glutamate excitotoxicity? Add a few examples of additional chemicals inducing excitotoxicity? Please make sure the Sidoryk-Wegrzynowicz 2013 reference is well formatted in the References section.

RESPONSE: References will be checked and corrected according to reviewer’s suggestion. Regarding excitotoxicity and calcium overload, this is mention in the specific AOP on excitotoxicity developed by Anna Price and coworkers and already endorsed by OECD.

ACTION: The reference of Fagnou was corrected. Chai et al., 2013 refers to trophic factors produced by astrocytes and is correctly cited here. The sentence about autism has been corrected according to reviewer’s suggestion. It is linked to the previous sentence. There is a lot of literature linking mercury and autism spectrum disorders in relation to glutamate dyshomeostasis, oxidative stress and neuroinflammation. We agree that this AOP is specifically on learning and memory, but it was just to give some broader framework.

P25, line2:

Concerning L-Glu transporters. The names of human L-Glu transporters and rodent homologues should be organized.

RESPONSE: We don’t understand what is requested here. cf additional information below:

Corrections EAAT2 not EAAT1 in method section will be done.

Addition of references to measure the activity of all glutamate transporters.

ACTION: Method section has been completed with the informations proposed by the reviewer including a table with the different inhibitors.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| human | rodent | distribution | Non-specific inhibitors | Specific inhibitors |
| EAAT1 | GLAST | Astrocytes, cerebellum,  High expression in astrocytes at developmental stage | TBOA  TFA-TBOA | UCPH |
| EAAT2 | GLT-1 | Astrocytes  >90% adult CNS L-Glu uptake | WAY213613  DHK |
| EAAT3 | EAAC1 | neurons |  |
| EAAT4 | EAAT4 | Purkinje cells |  |
| EAAT5 | EAAT5 | retina |  |

If you want to examine total L-Glu transport activities, you should first use non-specific L-Glu transporter inhibitors, like TBOA, TFB-TBOA, etc. If you want to focus on a specific subtype, you can try the specific inhibitors described above

RESPONSE: The non-specific and the specific inhibitors are mentioned in the table. We don’t understand what is requested here.

ACTION: Cf action above: a table with the different inhibitors has been added.

Page 29: Slight tweak to increase accuracy…”including volume regulation, and increased intracellular Ca2+ levels.”

RESPONSE: The page number did not correspond, therefore we don’t know what is referred here.

ACTION 2: “intracellular” was added in the KE description.

P30, line14-: Now a variety of markers have been suggested to be useful to distinguish between M1 and M2. In other words, microglial phenotype should be determined using a combination of multiple markers. I therefore recommend the following paper summarizing the markers for each microglial phenotype.

Moehle and West, Neuroscience 2015, 302, 59-73

RESPONSE: The reference will be added as suggested by the reviewer.

ACTION: The reference has been added as suggested.

P30, line 30: GFAP is also expressed in radial glia. You had better another astrocytic marker such as S100beta if you want to identify mature astrocytes.

RESPONSE: S100beta is already proposed as alternative marker of astrocytes.

NO ACTION REQUIRED

Appendix 1, KE 1492, p.32. I am assuming that KE 1492 Tissue Resident Cell Activation was developed for other AOPs and that the BRAIN subsection (which shares a lot with KE 188 Neuroinflammation) was added specifically for AOP 17. Once again, as for the MIE and first KE, the endpoint measured to assess KE 1492 and KE 188 are very similar. As previously explained this is a serious issue. The authors may want to inspire themselves of one sentence in the LIVER section: “KC activation and macrophage recruitment are two separate events and both are necessary for fibrogenesis, but as they occur in parallel, they can be summarised as one KE.” Please also note that while only the Nimmerjahn et al., 2005 citation was missing from KE 188, the Streit et al., 1999, Kraft and Harry 2011, Claycomb et al., 2013 Carson 2006, Aguzzi et al., 2016 and Rivest et al., 2009 citations are missing from the References section. Also, the first four publications listed in the References section are not cited in the text.

RESPONSE: In this AOP, we introduced the general KEs used to describe Inflammation in all tissues as it was discussed in the workshop. But in order to allow networking with the AOPs developed before the whorkshop, we introduced also the KE Neuroinflammation. We think that this compromise is specially important for the brain, where the concept of neuroinflammation is used in all publications. However, we should add a brain section in the KE “increased, pro-inflammatory mediators”.

ACTION: A text has been added in Background section at the beginning of the AOP to explain why we finally decided to keep “Neuroinflammation” as a KE and not replace it by the two KEs “Tissue Resident cell activation, and Pro-inflammatory-mediators, increased” as proposed in a workshop dedicated to Inflammation. The two main reasons to keep neuroinflammation as a KE are that Neuroinflammation is a concept recognized by the regulators and is found in the whole literature describing brain inflammation.

Appendix 1, KE 1493, p.36. Contrary to the previous KE there is no BRAIN subsection, only a LIVER subsection which is very specific to this organ. There are only a few sentences about brain right after Table 1. The Chao et al., 1995 reference is missing from the References section (where a few brain-related references were not cited in the text). Was the BRAIN subsection accidentally deleted? Was this an omission? The How is it Measured or Detected section is extremely vague. Is it because the authors wanted to avoid repeating the endpoints measured in KE 1492 and 188? Once again given that AOP are not meant to be exhaustive list of all the molecular mechanisms involved, but to be helpful guide for regulatory toxicology, the authors may want to reconsider the KE 188 1492 and 1493 to see if these could be lumped together or presented differently in order to ensure that different endpoints can be measured from different KEs.

RESPONSE: We will add a subsection about BRAIN.

ACTION: In fact, I am not sure that it is necessary to add a specific section on Brain, because the biomolecules are similar in all tissues, although their roles or importances may differ from one tissue to the other. I remember that we discussed that during the WS on representing the process of inflammation in AOPs. This is not the case for the other KE “Resident cell, activation”, where the cells involed in the inflammatory process differ from tissue to tissue.

Appendix 1, KE 1493, References p.39. Please check the spelling of Chen et al., 2009 (chng) and Kaimori et al., 2007 (Kaimorei) in the text, and the formatting of Shull et al., 2008 in the References section. The Korn et al., 2008 citation is not listed in the Reference section and the Calvin et al., 1992, Elyaman et al., 2008 and Zhou et al., 2008 references are not cited in the text.

RESPONSE: We will contact Brigitte Landesmann, who developed this part.

ACTION: Magda, could you contact Brigitte for these corrections?

ACTION 2: References removed as suggested by Brigitte.

P40, line 24: The information of the citation (Filippi et al.) is insufficient.

RESPONSE: We will contact Brigitte Landesmann, who developed this part.

ACTION: Magda, could you contact Brigitte for these corrections?

ACTION 2: Correction done by Brigitte.

Appendix 1, KE 386, How it is Measured or Detected, p.42. Please delete the first paragraph that just appears to be the instructions to fill this section.

RESPONSE: OK

ACTION: No action required. It was a problem on the snapshot.

Page 42: Tweak to increase accuracy “The ‘silent’ synapses disappear by PN 7-8 in rats and mice in both brain regions.”

RESPONSE: in rats and mice will be added as suggested by the reviewer.

ACTION: This KE has been developed by Anna Price and it is pretty evident that PN7-8 is in rodent. If it is in another species, it always specified. NO ACTION REQUIRED.

Page 42: Paragraph starting “There is strong evidence” – this paragraph needs better referencing of critical studies. Not all of the information in this paragraph was established by Henson, 2012.

RESPONSE: We agree, but it is not possible to be exhaustive. In addition, this KE was developed by Anna Price and coworkers and is shared by several AOPs, which underwent several processes of internal and external reviews.

NO ACTION REQUIRED

P42, line14:

The report by Henson et al. indicates that NR3A acts like dominant negative and synaptic maturation is regulated by the serial changes in the combination of NMDA subunits. In this original version, readers cannot understand the role of NR3A.

RESPONSE: We agree, but it is not possible to be exhaustive. In addition, this KE was developed by Anna Price and coworkers and is shared by several AOPs, which underwent several processes of internal and external reviews.

NO ACTION REQUIRED