August 9, 2024

To whom it may concern,

I declare that the review process for the AOP report titled "Development of an Adverse Outcome Pathway for Deposition of Energy Leading to Learning and Memory Impairment" and its accompanying AOPWiki entry (https://aopwiki.org/aops/483) was carried out according to OECD guidance for the scientific review of AOPs (OECD, 20211). All related documents are appended below. The review was conducted by the following committee members:

Review Manager:
Jason O'Brien

Reviewers:
Olivier Armant
Dmitry Klokov

Sincerely,

Jason O'Brien, PhD
Handling Editor, Environmental and Molecular Mutagenesis

OECD (2021). Series on Testing and Assessment No. 344: Guidance Document for the scientific review of Adverse Outcome Pathways. Organisation for Economic Cooperation and Development, Paris. Available at: https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm.

COACHES CHECKLIST AND REVIEW REPORT

ver. 2022-04-27

AOP Information

AOP number/title: 483, Deposition of Energy Leading to Learning and Memory Impairment

Author: Ahmad Sleiman, Kathleen Miller, Danicia Flores, Jaqueline Kuan, Kaitlyn Altwasser, Benjamin Smith, Tatiana Kozbenko, Robyn Hocking, Carole Yauk, Ruth Wilkins, Vinita Chauhan

Associated wiki page: *https://aopwiki.org/aops/483*

Compliance Reviewer Information

Name: Jason O'Brien

Organisation: Environmental and Molecular Mutagenesis

E-mail: jason.obrien@ec.gc.ca

Review Information

Date this checklist has been filled: 2023-06-05

Date of final draft PDF snapshot proposed for external review: 2023-06-05

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General Observations and Recommendations of the Reviewer

- Technically it is a small AOP network

KE ID	KE Title	Previously reviewed?	Which AOP?
1686	Deposition of Energy	YES	272
1392	Oxidative Stress	YES	17, 220
2066	Altered Signaling Pathways		
1492	Tissue resident cell activation		
2097	Increase, Pro-Inflammatory Mediators		
2098	Increase, Neural Remodeling		
1635	Increase, DNA strand breaks		
341	Impairment, Learning and memory		

KER ID	TITLE	ADJACENCY	Reviewed?	Which AOPs?
2769	Energy Deposition leads to Oxidative Stress	adjacent		
2832	Energy Deposition leads to Tissue resident cell activation	adjacent		
2771	Oxidative Stress leads to Altered Signaling	adjacent		
2833	Oxidative Stress leads to Tissue resident cell activation	adjacent		
2834	Tissue resident cell activation leads to Increase, Pro-Inflammatory Mediators	adjacent		
2835	Increase, Pro-Inflammatory Mediators leads to Increase, Neural Remodeling	adjacent		
2836	Increase, Neural Remodeling leads to Impairment, Learning and memory	adjacent		
2840	Altered Signaling leads to Increase, Neural Remodeling	adjacent		
2841	Increase, DNA strand breaks leads to Increase, Neural Remodeling	adjacent		
2811	Oxidative Stress leads to Increase, DNA strand breaks	adjacent		
1977	Energy Deposition leads to Increase, DNA strand breaks	adjacent	YES	272
2856	Increase, DNA strand breaks leads to Altered Signaling	adjacent		
2837	Energy Deposition leads to Increase, Neural Remodeling	non-adjacent		
2838	Energy Deposition leads to Impairment, Learning and memory	non-adjacent		
2839	Increase, Pro-Inflammatory Mediators leads to Impairment, Learning and memory	non-adjacent		

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Checklist

The following tables are checklists for the individual KEs and KERs and overal AOP

KE number, title: *1686, Deposition of Energy*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***272***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: • *Will ask authors to define components during scientific review*				

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KE number, title: *1392, Oxidative Stress*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***17 220***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?	*YES*			
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments:				

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KE number, title: *2066, Altered Signaling Pathways*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: • *Will ask authors to define components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title: *1492, Tissue resident cell activation*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***17 38***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?	*YES*			
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments:				

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KE number, title: *2097, Increase, Pro-Inflammatory Mediators*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?				*NO*
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?				*X*
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: • *Will ask authors about components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title:	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?				*NO*
Has the KE been reviewed by EAGMST?				*NO*
If an existing KE is being adapted, have the previous authors been informed?				*X*
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: *-will ask authors about components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title:	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***272 296***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?		*NO*		
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments: *-will ask authors about components during scientific review*				

AOP Coach Checklist and Final Review Report

KE number, title:	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KE already been used in other AOPs?	*YES*			
Has the KE been reviewed by EAGMST?	***12 13 17 48 54***			
If an existing KE is being adapted, have the previous authors been informed?	*YES*			
Is the KE described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is the biological context (level of organization, terms) specified?	*YES*			
Are KE components defined using structured ontology terms (Process, Object, Action)?	*YES*			
Is the KE description clear?	*YES*			
Are measurement methods specified, described and referenced?	*YES*			
Is the domain of applicability described?	*YES*			
Specific Comments:				

KEY EVENT RELATIONSHIPS

KER number, title: *2769, Energy Deposition leads to Oxidative Stress*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2832, Energy Deposition leads to Tissue resident cell activation*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2771, Oxidative Stress leads to Altered Signaling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2833, Oxidative Stress leads to Tissue resident cell activation*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2834 Tissue resident cell activation leads to Increase, Pro-Inflammatory Mediators*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2835 Increase, Pro-Inflammatory Mediators leads to Increase, Neural Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				*NO*
Has the KER been reviewed by EAGMST?				*NO*
If an existing KER is being adapted, have the previous authors been informed?				*X*
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2836 Increase, Neural Remodeling leads to Impairment, Learning and memory*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *2840 Altered Signaling leads to Increase, Neural Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *2841 Increase, DNA strand breaks leads to Increase, Neural Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *2811 Oxidative Stress leads to Increase, DNA strand breaks*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *1977 Energy Deposition leads to Increase, DNA strand breaks*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?	***272***			
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	*YES*			
Is biological plausibility described/discussed?	*YES*			
Is empirical evidence presented, referenced and discussed?	*YES*			
Are uncertainties and inconsistencies described?	*YES*			
Is Quantitative Understanding of the Linkage described?	*YES*			
Is Domain of Applicability described?	*YES*			
Specific Comments:				

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KER number, title: *2856 Increase, DNA strand breaks leads to Altered Signaling adjacent*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?	*YES*			
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?	*YES*			
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *2837 Energy Deposition leads to Increase, Neural Remodeling*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

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KER number, title: *2838 Energy Deposition leads to Impairment, Learning and memory*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

AOP Coach Checklist and Final Review Report

KER number, title: *2839 Increase, Pro-Inflammatory Mediators leads to Impairment, Learning and memory*	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Has the KER already been used in other AOPs?				NO
Has the KER been reviewed by EAGMST?				NO
If an existing KER is being adapted, have the previous authors been informed?				X
Is the KER described in a way that allows its reuse in other AOPs (i.e. independent of this AOP)?	YES			
Is biological plausibility described/discussed?	YES			
Is empirical evidence presented, referenced and discussed?	YES			
Are uncertainties and inconsistencies described?	YES			
Is Quantitative Understanding of the Linkage described?	YES			
Is Domain of Applicability described?	YES			
Specific Comments:				

AOP Coach Checklist and Final Review Report

OVERALL AOP

Overall AOP	*Yes*	*For revision*	*Revision agreed*	*Not applicable*
Does the title of the AOP follow the correct convention (MIE or first KE leading to AO)?	*YES*			
Does the title of the AOP reflect its content/domain?	*YES*			
Is a graphical representation included?	*YES*			
Is it clear who the authors/developers of the AOP are? Contact information for one or more corresponding author(s) should be included.	*YES*			
Is the status of the AOP described?	*YES*			
Does the abstract concisely describe the main content of the AOP in a standalone manner?	*YES*			
Have prototypical stressors been identified for the MIE?	*YES*			
Has the regulatory relevance of the AO been described?	*YES*			
Is the domain of applicability of the AOP defined in accordance with the OECD AOP Handbook?	*YES*			
Is the level of support for essentiality of the KEs described and assessed in accordance with the OECD AOP Handbook?	*YES*			
Has consideration been given to the level of support for the calls on the Overall WoE and the Quantitative Understanding?	*YES*			
Specific Comments:				

REVIEWER COMMENTS (ROUND 1 of 2)

REVIEWER #1

In their paper "Deposition of Energy Leading to Learning and Memory Impairment", S. Ahmad et al. propose an AOP for learning and memory impairment caused by exposure to radiations. The authors made a huge work and I would like to congratulate them already as this point, as they compiled a very large amount of data. The paper and the AOP they propose is very interesting, but several points need to be clarified. I hope these different points will further improve this nice work. My major concerns are following:

i) the applicability domain of this AOP must be clarified. First, if I agree with the MIE as deposition of energy, I do not agree with the inclusion of UV data in this AOP that apply on cognitive defects caused by ionizing radiation. Even for intermediate KE, inclusion of UV data is for me misleading as the process of ROS generation and DNA repair and damages are very different after UV and IR exposure, as well as the target cells (see for instance Ren Jie Tuieng Cells 2021). Second the evidences that ROS production and inflammation are occurring at dose < 1Gy is very weak. If the direct link between the MIE and the AO is well supported also at dose < 1Gy, the intermediate KE are not. The domain of applicability of this AOP is thus questionable (0.1 Gy to 1Gy and above). The mechanistic understanding of the adverse outcome through oxidative stress and pro-inflammation is thus also questionable at dose < 1Gy. All those points must be clarified and discussed in the paper.

ii) The KE neural remodeling is for me not appropriate, as well as the definition that authors propose for this term. Neural remodeling is usually considered as a process necessary for adaptation to adapt the brain to new information during development, learning and wound healing. It is thus not an adverse effect, but rather a process involved in the reorganization of the neural circuits either during learning or in response to changes in the environment. Some disease conditions are also leading to neural remodeling as in Parkinson and Alzheimer, but it is usually proposed as a way for the brain to compensate for neuronal function. It is thus usually not a cause of determent but a response. In addition the authors refer to neural and neuronal effects equivalently in the paper, which is wrong. The authors should clarify what is a detriment (senescence, apoptosis of neurons and glial cells, demyelination) and what is part of the healing process after exposure to ionizing radiation. Perhaps the authors can consider to differentiate papers describing effects like neuronal plasticity several weeks after exposure, as they are very likely to describe healing process and not necessarily an adverse effects. This is for me a major flow in this AOP.

iii) The KE altered signaling is not appropriate. This KE is so general that it can not be measure precisely and linked to a detriment. In their paper the authors propose this KE being related to defect in synaptic signaling and senescence (page 9 line 29 in the paper). But in the wikiAOP this is much less clear as it sometimes refer to synaptic activity, or differentiation, proliferation, apoptosis, survival. Such KE is so general and encompass so many processes that I do not believe it is useful in the AOP framework. Perhaps the authors could discriminate two different kinds of signalling to help organize better the complex changes occurring after damaging the brain: one process which is non cell autonomous (immune response for instance), another which is cell autonomous (synaptogenesis defect, cell death, cell senescence).

iv) The authors propose the Adverse Oucome as impaired learning and memory. If learning and memory are closely related concepts, the complexity of these functions and diversity of the

brain area they mobilise make the AO of this AOP very general. For instance what are the assays used to assess and discriminate these two functions? Are they the same? Is it possible that memory is impacted but not learning (and vice versa?)? I would like the authors to discuss this point on defining a single AO, while the complexity of cognitive function related to memory and learning is huge. This is for me a strong limitation of this AOP, as it is very difficult to claim that learning and memory are identical processes. I also would like to point that alteration of one brain area can have impact on others, which further blur the notion proposed by the authors that memory and leaning are supported by identical brain structure.

Other points:

-page 3 line 53: "However, data is currently lacking to utilize this approach to estimate risks to the CNS (Nelson et al., 2016; Miller et al., 2022)". The Miller reference is a review on the effects in the CNS but do not describe risks. I do not manage to find the Nelson paper, please provide a doi.

-page 4 line 8: "The existing animal and cellular data suggest significant adverse effects of space-relevant charged particles at low (<0.1 Gy) and high (4 Gy) ". Please provide a reference here about these doses.

Page 6 of the article. The authors make a very nice introduction on the cognitive effects caused by ionizing Radiation (IR) in human. They clearly demonstrate that data are missing at low and moderate dose compared to high dose (as it is very often the case). However I find it difficult to know if the proposed AOP, is applicable to all dose and dose rates, or only to high dose (1 Gy and above). This is especially important for the dose dependent effects related for instance to ROS, for which little evidence exist at dose < 1Gy. The authors cite different references provided page 11 line 51 ("Summary of Scientific evidence) of the article that describe ROS or RON increase. By looking at the different articles cited, one citation provide evidence that ROS can be increased at dose < 1 Gy : Baluchamy et al., 2012. The Baulch et al., 2015 and the Giedzinski et al., 2005 papers provide data showing increase in ROS at dose > 1Gy. The article De Jager, T. L., A. E. Cockrell, S. S. Du Plessis (2017) is on UV, for which I have difficulties to accept as an evidence for an AOP related to IR and space travel. The article from Rehman et al., 2016 does not show effects related to dose in Gy, and to my point of view can not be used to support ROS increase at dose < 1Gy. The citation Tahimic & Globus, 2017, does not provide support on the dose used to increase ROS production. The citation Wang et al., 2019 is a review on Heart diseases and does not provide much information on ROS related to the dose. And finally, the De Jager, T. L., A. E. Cockrell, S. S. Du Plessis (2017) is cited twice. At the end, the support that ROS increase is observed below 1 Gy is only supported by the Baluchamy et al., 2012. The same in the wikiAOP webpage. They propose an applicability domain to low and High LET and at 0.1 to 1Gy. But there is very little evidence that oxidative stress is similarly induced at 0.1 and above 1 Gy. Indeed the authors state in the empirical support (on the Wiki AOP webpage) "irradiated with protons at 1, 2, 5 and 10 Gy showed a dose-dependent increase in ROS levels (Giedzinski et al., 2005)." But the reference that similar effects are also happening at doses near 0.1 Gy is not provided. In the wiki AOP page ("Key Event Relationship Description) they cite the Du Plessis (2017) on UV, which is for me of low relevance in this AOP as it is not a type of radiation that can alter cognition or have neuronal effects. The Karimi et al., 2017 paper is describing effects on lens at 15Gy. In overall, to my point of view the authors, should clarified throughout the paper (and in the KER) how oxidative stress is increased at dose below 1 Gy, as evidence are currently relatively weak.

-Page 9 line 40. The authors should clearly precise that they focused on neurogenesis in the hippocampus. If not I do not know to which type of neurogenesis they are refering to.

-page 12 line 26: “and then induce neural remodeling through apoptosis”. This is for me an overstatement. Apoptosis is not a process involved in neural remodeling in adult brain after injury. Apoptosis can lead to neuronal death, and consequently synaptic plasticity can compensate for this loss. Or perhaps neurogenesis in very precise location like the hippocampus. In the naive adult hippocampus, new born neurons can undergo apoptosis (see for instance 10.1016/j.stem.2010.08.014), but this is not a process that drive neural remodeling itself in an injured brain. Neural remodeling is a rather consequence of apoptosis. I also find the term neural too vague here, as it can refer to both microglia, astrocyte and neurons.

-page 13 line 83: The cited reference provide evidence for increased ROS and proinflammatory signals in glia not in neurons.

-page 13 line 39 and below. The authors propose a KER as moderate between pro-inflammatory signals and neural remodeling. This term ‘neural remodeling’ is not supported by the provided literature. Indeed the authors provide information in this section on neuronal cells and their progenitors (for instance neurogenesis, and neuronal differentiation). The term neuronal remodeling usually refers to dendrites outgrowth and synaptic activity, not neurogenesis and neuronal differentiation. The term neural remodeling is encompassing both neuronal and glial cells, but microglia activation and effects of these cytokines is not a neural remodeling process. The term neural remodeling can be used to describe the process of brain regeneration, but it is not adequately used here to describe how ROS increased in the glial cells can affect neurons and their progenitors. The same is true in the next paragraph from page 14 line 36 to page 15 line 22 of the article. The authors describe the effects of inflammatory signals produce by microglia activation on neurons, dendrites, synpases connexion and neuronal progenitors (including effects on neurogenesis). This process is not a neural remodeling effects. It is a neuronal effects caused by inflammation in reaction to injury.

-I also question the authors about the evidence of inflammatory signals at dose < 1Gy. I do not find strong evidence that such process occurs at low dose.

-Page 15 line 28: “Neural remodeling refers to alterations through changes to neurogenesis, neurodegeneration, neuronal excitability and synaptic plasticity, demyelination and dendritic spine density”. I disagree with the usage of this term neural remodelling. The authors provide evidences on neurons and their progenitors and cite the Bálentová & Adamkov, 2020 for effects on astrocyte and oligodendrocytes. I think the link between microglia activation and neuronal effects is clear. It is also clear that IR can induce apoptosis of neuronal cells and astroglial cells. But there is no evidence to my point of view that neural remodeling is a cause that lead to impaired learning and memory. Neuronal death, astroglial cells death and induction of inflammation are causing neural damages that lead to the AO. But using the term neural remodeling as a KE that to the AO is not adequate, as neural remodeling is a process that is defined as the mobilization of neuronal and astroglial cells in reaction to damaged structure in order to repair them. I thus disagree with the definition provided by the authors that neural remodeling refers to “alterations”.

-page 16 line 34:’ although it involves alterations in the neural circuits that regulate these processes’. To which processes the authors are referring to? The phrasing is strange to me. It can be understood as ‘ alteration in neural circuits regulate inflammation and impair learning’.

-page 17 line 8: “that examined the effects of IR on the CNS, the doses ranged from 1 cGy to 10 Gy from”. The evidence at dose < 1Gy are very week for both ROS production and induction of inflammation. The direct impact from IR to impaired learning and memory is stronger and more convincing as proposed page 16 line 25, the cited references (Cekanaviciute et al., 2018; Kiffer et al., 2019b; NCRP, 2016; Pasqual et al., 2021. But I disagree with the statement ‘along with changes in antioxidant levels’ page 17 line 15, as these papers used page 16 line 25 are not providing any data on ROS increase. If papers that link IR at < 1Gy to ROS increase and the learning and memory deficit, they must be cited here.

-page 18 line 17: ‘neural remodeling following alteration of signaling pathways (El-Missiry et al., 2018; Chow et al., 2000; Suman et al., 2013; Kumar et al., 2005)’. I disagree with the term neural remodeling. These papers show effects on neuronal and glial compartments, like DNA damages but does not provide evidences of neural remodeling being responsible of the adverse effects.

-page 19 line 42:’ impaired learning and memory is not observed without exposure to stressors/insults.’ This is not completely true, since aging and diseases can also lead to the same outcome.

-page 21: ‘Modulating Factors’. The authors should cite age as modulator. In the process of aging increased stress and increases DNA damages or ROS contribute to the onset of learning and memory defects. See for instance psychological stress (10.1016/j.dr.2021.100968) or DNA damages and ROS (https://www.nature.com/articles/s41392-022-01251-0 + **https://doi.org/10.3390/antiox12030651**)

-page 22 line 42: “human-specific genes important for learning and memory such as Kallikrein-related peptidase 8”. It is not only novel gene expression that drive differences between human brain and other species. The authors should also cite other differences between primate and other mammalian species, such as the presence of oSVZ progenitor that drive much of the brain size differences with rodent for instance (https://pubmed.ncbi.nlm.nih.gov/25695268/)

-page23 line 83 : “The KE of neuronal remodeling has a wide range of endpoints. Neural remodeling encompass ». Neuronal remodeling and neural remodeling describe processes on different set of cell types. The authors should clarify throughout the manuscript this difference between “neural” and “neuronal”. I also do not agree with the proposition that neural remodeling is a KE, as proposed by the authors. The authors state ‘Neural remodeling encompasses changes to the physical and/or electrophysiological properties of neurons’ and then cite ‘demyelination, neurodegeneration, levels of neurogenesis, synapse formation/remodeling, synaptic activity and dendritic complexity’. The authors describe process that perturb neurogenesis, plasticity, myelination and neuronal electrophysiology. These processes can not be put together as “neural remodeling” which is rather used in to describe extensive remodeling of brain connectivity and regions either during developing or during injury like stroke. The term neural include both glia oligodendrocyte and neurons. The authors describe a series of event from inflammatory signal in microglia to neuron and astrocyte apoptosis and decreased neurogenesis. The process of neurite plasticity, synaptic outgrowth and reshaping brain connectivity are the processes to compensate for these loss, and not a KE that lead to the outcome. The term remodeling is thus not adequate to describe a detriment.

-Page 24 line 8: The authors state that ROS production and inflammation are barely observed at dose < 1Gy. The part the AOP with indirect KE leading to the AO, is thus not that simple in the domain of the low dose, despite clinical observation at < 1Gy in human. This is an interesting point, and I would like that this observation that KE ROS and inflammation are mostly observed at dose of 1Gy and

above appear clearly in the different parts of the paper and on WikiAOP, as for instance in the applicability domain as well as in the KE and KER descriptions.

-Page 25: “extensive research revealed that the three pathways lead to neural remodeling, which in turn is strongly linked to impaired learning and memory”. Same as before, I contest such definition as remodeling being a KE.

- KE and KER for resident cell activation and altered signaling pathways are separated in the proposed AOP. This mean that the authors propose that resident cell activation, that contribute to inflammation signals, is distinct from the KE altered signaling pathway. But in the overall Assessment (wikiAOP part Biological Plausibility), it is not clear which pathway belong to which KE as the authors describe” pro-inflammatory mediators and altered signaling pathways can lead to neural remodeling” including proinflammatory signals, senecesnce and apoptosis . Indeed the authors first describe inflammatory cytokines can affect neural remodeling and then state that “these cytokines act on different receptors to initiate several signaling pathways to induce neuronal degeneration, apoptosis or to propagate further pro-inflammatory responses”. They thus propose that proinflammatory signals are key to deregulated pathways like neuronal degeneration, apoptosis responsible for neural remodeling. I am thus not sure that the position of the KE 2066 on altered signaling pathways is appropriate. Time concordance of the different KE 2066, 1492 and 1493 is thus not clear.

-I also do not think that altered signaling pathway is a real KE. This is too vague to be measured. The authors pinpoint in the text (both in the wikiAOP and in the article) to several pathways that are pertinent. The KE 2066 must be better defined to describe a proper pathway related to specific cell of tissular effects. This link between KE 2066 and KE 2098 is too broad.

-In the wikiAOP I think the Applicability domain is justified only for rat, mice, human, dog, but not for the other species.

REVIEWER #2

KEY EVENTS

Key Event 1 (Molecular Initiating Event, MIE): 1686, Deposition of Energy
Note: Shared KE, Previously reviewed in AOP 272
Comments:
The depiction of energy deposition as the Molecular Initiating Event (MIE) is well-justified and have been discussed and agreed upon at several dedicated forums. However, it would be useful to clarify the specific mechanisms by which energy deposition initiates the cascade leading to oxidative stress. Attention to the types and sources of energy that are most relevant to this AOP would enhance its applicability. Excitation of molecules upon irradiation is also a deposition of energy - is it relevant to the KE? What % of deposited energy, e.g., upon gamma-irradiation, is excitation vs. ionization? And why excitation energy is not listed in Table 1 for the MIE?

Key Event 2: 1392, Oxidative Stress
Note: Shared KE, Previously reviewed in AOP 17 and 220
Comments:
This KE is fundamental in the AOP and is well-supported by empirical evidence. However, the current definition of KE 1392 is not sufficiently specific (even its description in Table 1 gives two very broad bullets, and not specific molecules or families of molecules involved). Although all specific molecules involved in the context of ionizing radiation are described in the texts and its various parts (IR), the KE seems to be too broad. Its ambiguity is also highlighted by opposite possible interpretations of down- and up-regulation of antioxidant enzymes. Within the context of this AOP it is understood that down-regulation only is a marker of oxidative stress. However, alone this readout is insufficient / indirect evidence for the presence of the oxidative stress. Besides, many authors interpret up-regulation of the anti-oxidant enzymes as oxidative stress, which is opposite to the logic used in the AOP 483.

Key Event 3: 2066, Altered Signaling Pathways
Comments:
The name of the KE should be consistent throughout the manuscript (e.g. it is called differently on the diagram and Table 1). The alteration of signaling pathways is a critical step in the AOP, linking initial oxidative stress to cellular responses. However, KE 2066 lacks specificity in describing altered signaling pathways. The human cellular system contains vast and complex network of signaling pathways in human cells, each with distinct roles and responses to external stimuli. It's accurate to state that all cellular functions are regulated by changes in signaling pathways. To enhance the precision and relevance of this KE, it is imperative to delineate the specific signaling pathways that are critically affected by IR and elucidate how these alterations drive the subsequent key event, KE 2098 - Increase in Neural Remodeling. While the manuscript and AOP address certain pathways with supporting evidence and their connection to neural remodeling, this crucial information is not readily apparent in the AOP or the KE description, potentially diminishing the AOP's utility by allowing critical details to be overlooked.

The description of this KE in Table 1 is overly broad and should focus on identifying key specific pathways instead of providing a generic definition of what a signaling pathway entails. Additionally, the 'how to measure' column in Table 1 lacks detailed guidance; the current information is insufficient for users aiming to measure the relevant molecules or changes experimentally. It would be more beneficial to include references to specific assays, kits, and methods associated with the molecules detailed in Fig2, thereby offering clear and actionable instructions for experimental measurement.

Key Event 4: 1492, Tissue resident cell activation
Note: Shared KE, Previously reviewed in AOP 17 and 38
Comments:
Tissue resident cell activation is indeed a pivotal event, particularly in the context of neuroinflammation. However, the term 'tissue resident cells' covers a broad range of cell types in different tissues. Probably, it would be beneficial to consider brain specific title of this KE abd to discuss the types of cells involved and their activation mechanisms in the context of IR exposure and the AO in question.

Key Event 5: 2097, Increase, Pro-Inflammatory Mediators
Comments:
This KE is wrongly given on the AOP diagram as KE 1493. The increase in pro-inflammatory mediators is a well-established response to tissue damage and stress. Similar to the previous KEs, however, the KE could include more specificity to provide more insights into what mediators or groups of mediators contribute to neural remodeling. It is extremely important to use literature that maintains the brain context, as it is well known that immune regulation is highly context-dependent and common pro-inflammatory mediators such as TNF-alpha and IL-6 can execute an anti-inflammatory function (Pro-inflammatory cytokine TNF-alpha as a neuroprotective agent in the brain - PubMed (nih.gov); Anti-inflammatory effects of tumour necrosis factor (TNF)-alpha are mediated via TNF-R2 (p75) in tolerogenic transforming growth factor-beta-treated antigen-presenting cells - PubMed (nih.gov); Defining the Role of Anti- and Pro-inflammatory Outcomes of Interleukin-6 in Mental Health - PubMed (nih.gov); Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke - PubMed (nih.gov)); examples for other molecules identified by the authors as anti-inflammatory factors can be found.
Perhaps, this KE spans both 'cell' and 'tissue' levels. At the cellular level, they are produced by individual cells in response to stimuli, while at the tissue level, their collective action and distribution- at a distance away from the original production and secretion - influence the overall inflammatory response within the brain tissue, impacting its function and health.

Key Event 6: 2098, Increase, Neural Remodeling
Comments:
Neural remodeling as a last step prior to the AO is a crucial KE. Much like with the previous KEs, however, the authors should make an attempt to provide more specificity to this KE definition. The manuscript should focus on detailing the mechanisms of neural remodeling in response to the previous key events. It would be beneficial to discuss how these changes directly lead to the adverse outcome of learning and memory impairment, paying a special attention to physiological characteristics of the key event, e.g., the brain domain specificity. Also, the manuscript should clearly define what is meant by 'neural remodeling' in the context of IR-induced effects. Neural remodeling can encompass a range of processes, from

synaptic plasticity and neurogenesis to dendritic pruning and changes in neural circuitry. The manuscript should specify which of these aspects are most relevant to IR exposure and the progression of this AOP. Such specificity will not only enhance the scientific validity of the AOP but also its applicability in predicting and managing IR-induced neurological effects.

The authors should consider changing the level from cell to tissue for this KE. Whereas some of the endpoints listed within this KE are indeed properties of individual cell, the KE, as defined refers t a tissue level process, the process by which the structure of the brain's neural networks is changed. Neural remodeling is a characteristic of both individual neurons and neural circuits within the brain tissue, reflecting the dynamic nature of the brain's architecture in response to internal and external stimuli.

Key Event 7: 1635, Increase, DNA strand breaks
Note: Shared KE, Previously reviewed in AOP 272 and 296
Comments:
It is somewhat surprising that even this, one of the first in the AOP Kes, lacks specificity. It is well known that single-strand breaks and double-strand breaks can lead to vastly different consequences and trigger very different signaling pathways. This KE should be revised to address this. The implications of specific DNA strand breaks in neurons and possibly glial cells, in particular, should be highlighted.

Key Event 8 (Adverse Outcome, AO): 341, Impairment, Learning and memory
Note: Shared KE, Previously reviewed in AOP 12, 13 17, 48, and 54
Comments:
As the adverse outcome, the impairment in learning and memory is the culmination of the AOP. The manuscript would benefit from, like with the 'neural remodeling', more attention to specific brain regions where the pathology occurs. The description of the AO may also address the potential for reversibility or mitigation of these effects.
Another suggestion is to consider whether the AO is a tissue vs. organism level effect. Most of the endpoints (if not all) listed within the AO (Fig 2) are characteristics of an organism, specifically of individuals who have the capacity for conscious recall of facts and events, cognitive capacities, etc. While they involve brain tissue, particularly regions such as the hippocampus and temporal lobes, memory and cognition as concepts refers to the function and capability of the entire organism to encode, store, and retrieve information consciously.

KEY EVENT RELATIONSHIPS

Adjacent KERs

2769, Energy Deposition leads to Oxidative Stress
Note: Shared KER,
Comments:
Decreased, as well as increased activities of antioxidant enzymes should not be equated to oxidative stress. Oxidative stress is assessed by direct measurement of the levels of ROS or RNS. If the authors postulate that decreased antioxidant enzymes are markers of oxidative stress (e.g. use of reference Klucinski et al 2008), then the authors may need to explain how studies showing the opposite fit in supporting this KER.

2832, Energy Deposition leads to Tissue resident cell activation
Comments:
This KER ought to be excluded from the AOP due to the absence of any identified biological mechanism that establishes a direct connection between the two KEs. It is through the preceding KEs that the MIE results in tissue resident cell activation.

2771, Oxidative Stress leads to Altered Signaling
Note: Shared KER
Comments:
This KER possesses significant biological plausibility, largely attributable to the expansive interpretation of 'Altered Signaling.' Essentially, any variation in gene expression or post-translational modification of proteins following oxidative stress can serve as corroborative evidence for this KER. Consequently, assessing this KER is challenging without first narrowing down the definition of the Key Event "Altered Signaling." For example, dose and time concordance will greatly depend on what readout is used for altered signaling and those readouts can be early and late type of responses to external stimuli.

2833, Oxidative Stress leads to Tissue resident cell activation
Comments:
For this reviewer, it is unclear how tissue resident cell activation could proceed without the involvement of signaling cascades or pathways as effectors. This perspective stems from the widely accepted understanding of cellular response mechanisms to stimuli, which involve signaling processes. Therefore, it is strongly advised to contemplate the exclusion of this KER from the AOP.

2834, Tissue resident cell activation leads to Increase, Pro-Inflammatory Mediators
Comments:
The link between tissue resident cell activation and pro-inflammatory signaling is well recognized in the scientific community. However, the authors should focus on citing studies that are directly relevant to the experimental models under consideration. It is questionable whether findings from, for instance, mouse kidney (Scharpfenecker et al., 2012) or human monocytic leukemia cell lines (Lodermann et al., 2012), can substantiate this KER, given its specificity to particular tissues and contexts. Therefore, it is advised that the authors refine

the references cited for this KER, a task that would be simplified by redefining the KE as previously suggested.

2835, Increase, Pro-Inflammatory Mediators leads to Increase, Neural Remodeling
Comments:
This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

2836, Increase, Neural Remodeling leads to Impairment, Learning and memory
Comments:
This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of Neural Remodeling.

2840, Altered Signaling leads to Increase, Neural Remodeling
Comments:
This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

2841, Increase, DNA strand breaks leads to Increase, Neural Remodeling
Comments:
To this reviewer, the direct progression from DNA breaks to neural remodeling without intermediate biological events is unclear. Notably, even early neural remodeling endpoints, such as cell death, necessitate the involvement of various pathways, including DNA repair, cell cycle regulation, and apoptosis, all of which are mediated by signaling cascades. Consequently, it is advised that this KER be reconsidered for inclusion in the AOP due to the necessity of these intermediate steps.

2811, Oxidative Stress leads to Increase, DNA strand breaks
Note: Shared KER
Comments:
This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

1977, Energy Deposition leads to Increase, DNA strand breaks
Note: Shared KER, Previously reviewed in AOP 272
Comments:
No comments

2856, Increase, DNA strand breaks leads to Altered Signaling
Note: Shared KER
Comments:
This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

Non-adjacent KERs

2837, Energy Deposition leads to Increase, Neural Remodeling
Comments:
It is not clear how this KER should be part of the AOP since the whole purpose of the AOP is to generate a causally and mechanistically linked chain of biological key events; from early to late events, such as these two KEs. Thus, positing a direct linkage between them appears implausible, suggesting a reevaluation of their inclusion is warranted for coherence with the AOP's foundational principles.

2838, Energy Deposition leads to Impairment, Learning and memory
Comments:
Same as for KER 2838

2839, Increase, Pro-Inflammatory Mediators leads to Impairment, Learning and memory
Comments:
Same as for KER 2838

OVERALL AOP PAGE

Comments:

1. The "AOP 483 snapshot" document outlines the development of an AOP related to the deposition of energy leading to learning and memory impairment. It details the sequence of key events starting from the MIE of energy deposition, through various biological processes such as oxidative stress, altered signaling pathways, tissue resident cell activation, and increased pro-inflammatory mediators, leading to neural remodeling. The culmination of these events results in the adverse outcome of impaired learning and memory. The document provides a comprehensive overview of each key event, including their biological basis and interconnections within the pathway. The authors carried out a robust review of literature using a modified systematic review approach and should be commended for an outstanding effort. The resulting AOP is a significant advancement in the field. However, given the breadth of the scope (space/cosmic radiation and other radiation types) and the complexity of the multifactorial adverse outcome relevant to behavioral changes, the AOP would benefit from further revision that would address comments and concerns expressed in this review.

2. The generic nature of the key events used by the authors makes it very difficult to access the aspects of the AOP such as empirical support. Indeed, the authors themselves Refer to studies that measure specific markers such as P53 BAX, BCL-2 etc. But insufficient specificity is provided for tissue remodeling or altered signaling pathways: *"Few studies showed incidence concordance where the upstream KE demonstrated a greater change than the downstream KE following a stressor. Not all KERs displayed an incident-concordant relationship, but for those that did, only a small proportion of the empirical evidence supported this relationship. For example, mice exposed to 2 Gy of gamma irradiation showed increases of pro-apoptotic markers p53 and BAX by 8.4- and 2.3-fold, respectively. A 0.6-fold decrease in Bcl-2 (anti-apoptotic marker) was also observed, and gamma rays cause a decrease in cortical thickness by 0.9-fold (Suman et al., 2013)."* This is an example how the use of generic key events undermines the utility of the AOP concept.

3. The lack of positive and negative feedback loops in the AOP significantly undermines the process' complexity and regulatory intricacies. Positive feedback mechanisms, such as the induction of ROS by pro-inflammatory responses, as well as negative compensatory circuits, such as induction of anti-oxidant enzymes upon altered signaling pathways, are critical for understanding and describing the progression of neurological damage. This oversight simplifies the dynamic and interconnected nature of brain responses, potentially leading to inaccuracies in predicting the severity and progression of radiation-induced cognitive impairments. Incorporating these loops is essential both for scientific accuracy and for enhancing the predictive accuracy of the AOP and applicability (e.g.., guiding effective interventions, risk prediction, etc.).

4. The authors should specifically consider a feedback loop from a pro-inflammatory mediators secreted by neural resident cells to alterations in signaling pathways. This would highlight the intricate relationship between inflammation and signaling pathway modulation within the brain.

5. Page 21: Correct “UVC radiation (X-X nm)”

6. Referencing studies on ultraviolet (UV) radiation effects, such as de Jager, Cockrell, and Du Plessis (2017), which explore the impact of UV on antioxidant enzymes, does not seem appropriate. This is because UV radiation primarily affects the skin and does not penetrate deeply enough to directly impact brain tissues or functions.

AOP REPORT MANUSCRIPT

Comments:

1. Issue with the generic nature of many key events is exemplified by the KE 1493: pro-inflammatory mediators can exhibit anti-inflammatory effects under certain conditions. This paradoxical role well known and is a part of the complex and dynamic nature of the immune system. The function of pro-inflammatory mediators can be context dependent. Factors such as concentration, timing, and the specific microenvironment can influence whether a mediator acts as pro-inflammatory or anti-inflammatory. Some pro-inflammatory mediators play roles in resolving inflammation. For instance, certain types of prostaglandins, initially promoting inflammation, later contribute to the resolution phase. The immune system has feedback mechanisms where prolonged inflammation leads to the activation of anti-inflammatory pathways. Some cytokines, like IL-10, have dual roles in both promoting and inhibiting inflammation. Cytokines may switch roles by modulating signaling pathways. For example, TNF-α is primarily pro-inflammatory factor, but can induce anti-inflammatory effects under specific conditions. In some cases, mediators that cause inflammation in one tissue may have anti-inflammatory effects in another. The interaction of pro-inflammatory mediators with other molecules in the immune system can modify their effects, leading to anti-inflammatory outcomes (Serhan and Savill (2005). Resolution of inflammation: the beginning programs the end. Nature Immunology, 6(12), 1191-1197; Lawrence and Gilroy (2007). Chronic inflammation: a failure of resolution? International Journal of Experimental Pathology, 88(2), 85-94; Aoki and Narumiya (2012). Prostaglandins and chronic inflammation. Trends in Pharmacological Sciences, 33(6), 304-311; Smith et al. (2000). Cyclooxygenases: structural, cellular, and molecular biology. Annual Review of Biochemistry, 69, 145-182; Nathan and Ding (2010). Nonresolving inflammation. Cell, 140(6), 871-882; Opal and DePalo (2000). Anti-inflammatory cytokines. Chest, 117(4), 1162-1172). These are all possible scenarios upon IR exposure of the brain and the lack of specificity and detail in the AOP 483 undermines its utility in hypothesis generation and knowledge gap identification.

2. Lack of discussion with respect to what brain regions are involved in each KE/KER; it is known that damage to different brain domains can impact learning and memory in distinct ways. The brain is a complex organ with various regions responsible for different aspects of learning and memory. Brain domains such as hippocampus, frontal lobes, temporal lobes, parietal lobes, cerebellum and basal ganglia, amygdala all have distinct roles in learning and memory (The right parietal lobe is critical for visual working memory - PubMed (nih.gov); Human emotion and memory: interactions of the amygdala and hippocampal complex - PubMed (nih.gov)) This should at least be discussed,

and ideally evidence from IR studies or studies covering the KER of the AOP should be presented.

3. One aspect that the authors should consider including in the revised manuscript is the assessment of the relative amount of evidence that is supporting this AOP (positive evidence) vs. the evidence that is non-supporting (negative evidence). It is hoped that the literature screening and data extraction approach used by the authors would allow to carry out such assessment. This information appears to be very important for the identification of knowledge gaps and inconsistencies in a quantitative manner. Just as an example, a study by Chien et al (Low-dose ionizing radiation induces mitochondrial fusion and increases expression of mitochondrial complexes I and III in hippocampal neurons - PMC (nih.gov) could be mentioned where the finding suggest compensatory mechanisms at low, but not high dose of IR. Including such evidence in the assessment seems crucial: those KEs and KERs that would have the lowest ratio [positive/negative] or have low absolute number (not %) of positive evidence papers would be immediately tagged as knowledge gaps. Furthermore, this information, if related to the dose range, life stage and taxonomic applicability (shown in Fig 5) can provide unprecedented level of understanding of the relevance of biological mechansisms to human radioprotection scenarios (high vs. intermediate doses) and would inform future studies.

4. Fig 5: How the data shown were calculated? And how they are distributed over the KERs? It would also be interesting to see somewhere in the AOP and the manuscript the number of included supporting studies that a) were done using non-IR stressors/treatments and b) were done in non-neuron/brain related models.

5. Fig. 6: There are several questions here: a) why some parts do not have low dose label; how to find them, they are not in order (low-intermediate-high) for each section? B) what are unlabeled zones (question marks on the screen shot below)?

6. Suggestions for Table I :
   Consider adding the following methods for KE 1392: a) Chemiluminescence: This method involves luminescent probes that emit light when they react with ROS. The light intensity is proportional to the ROS level, providing a direct measure of oxidative stress. B) Electron Spin Resonance (ESR) Spectroscopy: This technique directly detects free radicals by measuring their unpaired electrons using magnetic fields and radiofrequency. It's considered the gold standard for direct free radical measurement.

7. Page 24, lines 3-9: It seems that this uncertainty applies to many other parts of the AOP and may not be listed under bullet #2

8. Page 24, lines 23-34: This uncertainty/inconsistency should be extended to KEs 2066, 1492 and 1493. They also have a wide range of readouts and markers that can be used to define them.

9. Page 29: sentence “the AOP could be part of the literature evaluation used to consider the reclassification of health effects from radiation exposures” should be clarified

AUTHORS RESPONSES TO REVIEWER COMMENTS (Round 1)

Thank you for your thoughtful review of our adverse outcome pathway (AOP) manuscript and associated documents. We appreciate the time and effort you have dedicated to providing very constructive feedback. We have reviewed the comments and where appropriate have addressed them as outlined below.

Some general points to consider when reviewing our responses:

- Most KEs (new KEs include #2066 & 2098) in our AOP are reused from existing endorsed AOPs in the AOP Wiki. This also includes the AO. Thus, this limits the extent of changes that can be made to them. AOPs are built in a modular fashion to ensure that KEs and KERs are shared between AOPs. This is a core principle that we are required to adhere to in AOP development so that networks can emerge in the future.

- KERs are modular units and independent from the rest of the AOP; therefore, they are also supported by data derived from different cell types and organs as they may be relevant to multiple AOs. Thus, some KERs are supported by data from non-brain cells and non-ionizing radiation, and we cannot change this.

- KE descriptions are intended to be brief, simply describing the relevant key structural and functional aspects of the KE that allow for its measurement. KEs are applied to multiple AOPs, and discussion on downstream and upstream events is not described within KE descriptions (they would no longer be modular). Therefore, this information is thus in the KER or overall AOP descriptions. Indeed, the OECD would not endorse our AOP if the KEs referenced other KEs or KERs, as it would violate the principle of modularity. Where applicable, we have expanded the descriptions of the KEs.

- Qualitative AOPs can be supported by a wide range of radiation stressors and therefore, our AOP is not specific to any particular exposure parameter (i.e., dose, dose range, or radiation quality). Our interest is on understanding the upstream biological perturbations in the context of the downstream KEs.

- Not all dose ranges support each KER in the AOP. Most of the KERs are supported by moderate to high dose data (Figure 5) and a few early macromolecular events are supported by low dose data.

- Some KEs are broad in scope as multiple measurements are needed to assess the impact on downstream events and the current state of knowledge does not allow focus on one aspect of the KE. As more knowledge emerges, these KEs can be split into more specific KEs. This is the strength of AOPs, which are 'living

documents' stored in wiki format that can be updated when new data or tests emerge. It is our intent to manage these AOPs in this manner, with regular updates as science progresses.

- Non-adjacent KEs are important to include in an AOP. This inclusion of data is particularly valuable as it contributes to reinforcing the weight of evidence for the overall AOP since some KEs are not routinely measured. Additionally, the presence of multiple MIEs leading to KEs throughout the AOP aids in strengthening a quantitative understanding of the pathway. Given the often limited quantitative data available for adjacent relationships, the incorporation of non-adjacent relationships in AOPs becomes essential to address this gap.

General Responses to Main Comments:

The following are summaries of the main concerns highlighted by the reviewers.

Broadness of scope of KEs

We acknowledge the broad nature of a few KEs in our AOP. We emphasize that a number of these are reused from endorsed AOPs (highlighted in Figure 1 of the AOP report). While we understand the importance of specificity, our chosen KEs are broad to allow reuse and reflect the current state of available evidence. The good news is that AOPs are not static – they can be modified as new evidence emerges. Moreover, in Figure 2 of the AOP report, we provide the predominant and specific endpoints that informed our AOP. Figure 2 summarizes the specific measurable endpoints that contributed to the development of our pathway (despite the generic name of the KE).

Definition of neural remodeling

We debated extensively about this within our team and have decided to retain this KE name but add "abnormal" to it. The KE has been changed to "abnormal neural remodeling" Abnormal neural remodeling can encompass dendrite outgrowth, decreased synaptic activity, decreased neurogenesis, and decreased neuronal differentiation. This is discussed in several reviews that are cited in the AOP: Cekanaviciute et al., 2018; Hladik & Tapio, 2016; Kiffer et al., 2019b; and Makale et al., 2017. As highlighted within the KERs linked to abnormal neural remodeling, the studies we retrieved show evidence related to morphological changes in neural cells (e.g., decrease in dendritic complexity/spine density, and demyelination) altered functional properties defined by decreased synaptic plasticity and neurogenesis) and altered communication. This also includes neuronal death, astroglial cell death, and induction of inflammation as these could also be associated with maladaptive neural remodeling. In the context of maladaptation, these processes may represent aberrant or harmful changes in neural structure and function, leading to negative consequences for overall neural network integrity and cognitive function. Abnormal neural remodeling may result from various factors, including radiation exposure, and it contrasts with the typical adaptive changes associated with neural plasticity and remodeling.

Some examples of studies used to support the KER of neural remodeling to learning and memory impairment include:

- Neurogenesis:
  - Reduced neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) of hippocampus is associated with impaired learning and memory (Bálentová & Adamkov, 2020; Monje & Palmer, 2003).
  - Decreased neurogenesis correlates with hippocampal-dependent cognitive dysfunction (Tomé et al., 2015).
- Neurodegeneration:
  - Apoptosis leads to o neurodegeneration which negatively impact cognitive function (Bálentová & Adamkov, 2020; Hladik & Tapio, 2016).
  - Hippocampal atrophy corresponds to the degree of impaired learning and memory (Tomé et al., 2015).
- Synaptic Plasticity and Excitability:
  - Synaptic strength, neuronal excitability, and long-term potentiation (LTP) are crucial for learning and memory (Romanella et al., 2020).
  - Decreased hippocampal excitability and disrupted LTP are associated with reduced learning and memory (Romanella et al., 2020).
  - Changes in synaptic receptor expression and other synaptic proteins contribute to impaired learning and memory (Hladik & Tapio, 2016).
- Demyelination and White Matter Necrosis:
  - Demyelination is linked to decreased long-term memory formation (Tomé et al., 2015).
  - White matter necrosis, along with demyelination, leads to impaired learning and memory (Bálentová & Adamkov, 2020).
  - Sub-threshold demyelination cause learning and memory deficits (Monje & Palmer, 2003).
- Dendritic Spine Density and Complexity:
  - Studies demonstrate reduced dendritic branching, length, and area in hippocampal neurons associated with learning and memory deficits (Hladik & Tapio, 2016).
  - Reduced dendritic complexity and spine density are associated with impaired learning and memory (Bálentová & Adamkov, 2020; Hladik & Tapio, 2016; Romanella et al., 2020).
  - Loss of dendritic spines in the hippocampus results in reduced signal processing and impaired learning and memory (Romanella et al., 2020).

The various aspects of neural cell function and structure (collectively neural remodeling) predominantly in hippocampus region of the brain have been shown to result in learning and memory impairment in animal models using tests such as fear conditioning (FE), object in place (OiP), delayed matching to sample (DI) and novel object recognition (NOR). More details can be found within the KER of neural remodeling to learning and memory impairment.

Furthermore, we were not confident to focus on a specific aspect of these two (functional and structural) varied neural level changes; the scope of the KE was selected to reflect the broadness of available data. The evidence to support the KER is derived from both neurons and glial cells; therefore, we feel that "neural" cells is the appropriate terminology. We cannot confidently say that learning and memory impairment is exclusively from one specific cell type. There could be many ways learning and memory impairment can be initiated. Thus, we feel that capturing the current state of knowledge with a wider lens is more prudent at present than focusing on one specific aspect that is only supported by limited data with uncertainties related to measurements and meeting the stringent Bradford Hill criteria.

Rationale for inclusion of signaling pathways in AOP

We can appreciate the criticism of the broad name assigned to this KE at present. However, although the KE name is generic, the data presented within the KERs is sufficiently specific to justify the connections to downstream events in the AOP. Because the KE encompasses several signaling pathways that are occurring in parallel, there was no way to identify a single non-generic 'name' for the KE that quite captured it. Breaking the KE up into multiple small KEs is overly complicated (does not adhere to best practices in AOP development) and the signaling pathways are all inter-connected.

Despite the broad name, we have explicitly highlighted the involvement of critical signaling pathways such as the cAMP-PKA (cyclic adenosine monophosphate-protein kinase A) pathway, the MAPK (mitogen-activated protein kinase) pathway, and the PI3K-Akt (phosphoinositide 3-kinase-protein kinase B) pathway in the AOP. These pathways are well-documented in the literature for their roles in synaptic plasticity, neuronal survival, and memory formation. Details can be found within the KER description of signaling pathways to neural remodeling and also summarized in Figure 2 of the AOP report. Thus, although the name is broad, the details are provided within the KE and KER descriptions, and we think this captures what is most relevant.

Rational for the inclusion of proinflammatory mediators

The inclusion of the pro-inflammatory mediators KE (reused from the OECD endorsed AOP "Oxidative stress and Developmental impairment in learning and memory" AOP 17; https://aopwiki.org/aops/17) in our AOP is also supported by moderate empirical evidence. Specifically, the KE describes how alterations in cytokines such as TNF-α, IL-1β, and IL-6, are well established to occur in empirical studies. Animal models have consistently shown that elevated levels of these mediators inhibit neurogenesis, impacting the formation of new neurons in regions critical for learning and memory, such as the hippocampus. Additionally, in vitro experiments elucidate the direct effects of these mediators on neural progenitor cells and their differentiation. The empirical data presented within KERs also provides mechanistic understanding of how neuroinflammation contributes to learning and memory impairment.

Applicability of the AOP to low doses

We agree that it is important to be clear on the fact that few studies examine low dose effects; most studies assess effects at moderate to high doses. We have made this clearer, as detailed below. We include clearer statements on this in the AOP report (uncertainty section), overall

assessment, and the KER of "deposition of energy to oxidative stress". In addition, Figure 5 within the AOP report summarizes the dose ranges used to support the AOP and from the summary it is clear the very few low dose studies were available.

Detailed Response

Note: Responses in red are our general replies and responses in blue are specific changes made to the snapshot or/and AOP report.

REVIEWER #1

Comment: In their paper "Deposition of Energy Leading to Learning and Memory Impairment", S. Ahmad et al. propose an AOP for learning and memory impairment caused by exposure to radiation. The authors made a huge work and I would like to congratulate them already as this point, as they compiled a very large amount of data. The paper and the AOP they propose is very interesting, but several points need to be clarified. I hope these different points will further improve this nice work. My major concerns are following:

Reply: We appreciate this positive feedback.

Comment: The applicability domain of this AOP must be clarified. Second the evidences that ROS production and inflammation are occurring at dose < 1Gy is very weak. If the direct link between the MIE and the AO is well supported also at dose < 1Gy, the intermediate KE are not. The domain of applicability of this AOP is thus questionable (0.1 Gy to 1Gy and above). The mechanistic understanding of the adverse outcome through oxidative stress and pro-inflammation is thus also questionable at dose < 1Gy. All those points must be clarified and discussed in the paper.

Reply: The AOP is qualitative in nature, it is not intended to be specific to any dose, dose-rate, or radiation quality. Within the AOP report, although we provide information on the stressors that have supported the AOP, not all of these exposure parameters inform every KER in the AOP. In other words, some KERs may be enriched with data from moderate dose exposures vs others at higher doses. Some data was found at low doses e.g., within KER of deposition of energy leading to oxidative stress there are studies represented to low doses (<0.1 Gy) such as Baulch et al., 2015; Tseng et al, 2014; Veeraraghan et al., 2011; Baluchamy et al., 2012. Nonetheless we do highlight the uncertainty in low dose data in the overall assessment, AOP report and the KER of deposition of energy leading to oxidative stress. However, as the information on the inclusion of dose in the overall assessment has created confusion, we have revised to clarify this point as described below.

The paragraph in the overall assessment of the snapshot reads as follows on page 5 of the snapshot:

"This AOP was derived from data that investigates the CNS of humans, animals and cellular models following predominantly exposure to ionizing radiation. The AOP is qualitative in

nature and not intended to be specific to any particular exposure parameter. The exposure parameters informing the AOP include doses of moderate-high (>1 Gy) and both high and low-LET radiation qualities. However, the extent to which cognitive deficits exist at low-to-moderate ionizing radiation doses (0.1 Gy - 1 Gy) across all the KEs in the AOP remains incompletely understood as limited empirical evidence was retrieved to support this understanding."

AOP report pg 26

"A large amount of uncertainty surrounds the impact of low-dose IR on the occurrence of KEs, especially tissue resident cell activation and oxidative stress. Several studies found unexpected effects of low-dose radiation on oxidative stress. For example, low-dose neutron radiation increased the activity of antioxidant enzymes glutathione (GSH) and SOD, and the concentration of malondialdehyde, a product of oxidative stress, decreased (Chen et al., 2021). .Changes in antioxidant enzymes was observed in the rat lens, levels in the brain were not studied. More evidence is required to determine the relationship between IR at doses <1 Gy and tissue-resident cell activation. "

Within KER deposition of energy leading to oxidative stress on page 83 of snapshot:

"There is limited data to support an understanding of deposition of energy leading to oxidative stress at low doses."

Comment: First, if I agree with the MIE as deposition of energy, I do not agree with the inclusion of UV data in this AOP that apply on cognitive defects caused by ionizing radiation. Even for intermediate KE, inclusion of UV data is for me misleading as the process of ROS generation and DNA repair and damages are very different after UV and IR exposure, as well as the target cells (see for instance Ren Jie Tuieng Cells 2021).

Reply: AOPs are stressor agnostic. Some early macromolecular KEs (e.g., oxidative stress, tissue resident cell activation, DNA strand breaks) are relevant to non-ionizing and ionizing radiation. Therefore, where data meeting Bradford Hill criteria was available to support the relationship, irrespective of the stressor (as per OECD guidelines), it was included. Furthermore, the AOP was built with the intent to include the multitude of radiation stressors that would be encountered during space travel. Although non-ionizing radiation does not pose the same immediate health risks as ionizing radiation for astronauts, due to shielding, it is a stressor that is relevant to the space environment with UVC being particularly penetrative and mutagenic. All forms of UV can initiate the production of free radicals which can initiate pro-inflammatory mediators. A collective combination of stressors can lead to sufficient oxidative stress to overwhelm protective mechanism, thereby initiating downstream KEs in the AOP (e.g. DNA strand breaks). Therefore, including UV studies in the AOP is required under the OECD AOP principles.

However, in light of the reviewers' comments, we highlight this concern. We now have added the following to the snapshot on page 4 and 5:

"Since KERs are independent units from the rest of the AOP and can support multiple AOs, some macromolecular KERs may include studies from cell types (e.g., lens cells) and stressors (e.g., UV) not directly relevant to the AO."

AOP report page 11:

"Although the AOP is mostly supported by IR studies, a few studies relate to non-ionizing exposures, specifically macromolecular-level KERs. The collective impact of various stressors, including ultraviolet exposure, can include the generation of free radicals and then initiate downstream pro-inflammatory mediators. Since AOPs are stressor-agnostic, this collective burden (i.e., multiple stressor exposure) may overwhelm protective mechanisms, thereby triggering further KEs along the AOP."

Comment: The KE neural remodeling is for me not appropriate, as well as the definition that authors propose for this term. Neural remodeling is usually considered as a process necessary for adaptation to adapt the brain to new information during development, learning and wound healing. It is thus not an adverse effect, but rather a process involved in the reorganization of the neural circuits either during learning or in response to changes in the environment. Some disease conditions are also leading to neural remodeling as in Parkinson and Alzheimer, but it is usually proposed as a way for the brain to compensate for neuronal function. It is thus usually not a cause of determent but a response. In addition the authors refer to neural and neuronal effects equivalently in the paper, which is wrong. The authors should clarify what is a detriment (senescence, apoptosis of neurons and glial cells, demyelination) and what is part of the healing process after exposure to ionizing radiation. Perhaps the authors can consider to differentiate papers describing effects like neuronal plasticity several weeks after exposure, as they are very likely to describe healing process and not necessarily an adverse effects. This is for me a major flow in this AOP.

Reply: We appreciate the reviewer's concern. Endpoints (e.g., neurogenesis, alteration in neural structures, demyelination, etc.) associated with neural remodeling were assessed concurrently to downstream behavioral changes representative of learning and memory impairments (see detailed studies in KER of neural remodeling to learning and memory impairment). This includes test methods that examine in animal models decline in associative, discriminative and reversal learning and also show decreased memory (eg. spatial, working and declarative). Therefore, based on the evidence we have identified, we believe the KE is appropriately named. It was important to have an event in the AOP that is representative of neural level changes. The studies we retrieved show evidence related to morphological changes in neurons (e.g., decrease in dendritic complexity/spine density, and demyelination) and altered functional properties defined by decreased synaptic plasticity and neurogenesis. All these events can be defined as neural remodeling and there is evidence that this may lead to improper neuronal connections which underlie many brain diseases (Yaniv & Schuldiner,

2016; https://doi.org/10.1002/wdev.241). We were not confident to focus on a specific aspect of these two (functional and structural) varied neural level changes; therefore, the scope of the KE needed to reflect the broadness of available data.

With respect to the reviewer's concern on the neural remodeling being adaptive changes, we agree, that could be a possibility, but these changes could be maladaptive and the studies we retrieved show downstream detriments to learning and memory.

In light of the reviewers comment we have revised the name to “abnormal neural remodeling” added clarification on why the neural remodeling KE is broad and discuss the adaptability aspects of neural remodeling within AOP report and overall assessment as follows:

Overall assessment on snapshot page 5:

“While neural remodeling is a natural process that allows the brain to continue to adapt, long-term exposure to stressors such as the space environment (e.g., microgravity and space radiation) may lead to chronic inflammation and possible changes in structure and function of neural cells ultimately resulting in cognitive deficits. The progression of KEs along the proposed hypothetical AOP is driven by persistent oxidative stress and chronic release of pro-inflammatory markers, creating an environment of neuroinflammation.”

Page 8 of snapshot:

"The scope of several KEs in this AOP is broad and this reflects a level of uncertainty in exact endpoints that specifically link to the AO; therefore, several KEs (e.g. neural remodeling and signaling pathways) are defined by multiple structural and functional measurements.”

AOP report page 10 and 11:
“Note, that the scope of some KEs (signaling pathways and neural remodeling) is broad as multiple measurable endpoints were used to support the empirical relationship, in order to represent better the current state of knowledge and meet stringent Bradford Hill criteria.”

“Neural remodeling includes changes in the morphological properties of neural cells as well as altered functional properties such as impaired neurogenesis and neurodegeneration occurring in the hippocampus. Although neural remodeling is typically a beneficial and ongoing process that enables the brain to adapt, certain stressors such as the space environment may lead to maladaptation's, potentially resulting in cognitive deficits despite the brain's continued efforts to adjust.”

AOP report page 24 and also highlighted in Table IV:

The KE of neural remodeling has a wide range of endpoints. Neural remodeling encompasses changes to the physical and/or electrophysiological properties of neurons. Several endpoints are usually measured/analyzed for the KE such as demyelination, neurodegeneration, levels of

neurogenesis, synapse formation/remodeling, synaptic activity and dendritic complexity (spine number and density). Variations between protocols in different studies are the main source of inconsistency. The scope of neural remodeling could be refined and more precisely delineated in terms of specific endpoints once a definitive mechanism is identified.

Comment: The KE altered signaling is not appropriate. This KE is so general that it can not be measure precisely and linked to a detriment. In their paper the authors propose this KE being related to defect in synaptic signaling and senescence (page 9 line 29 in the paper). But in the wiki AOP this is much less clear as it sometimes refer to synaptic activity, or differentiation, proliferation, apoptosis, survival. Such KE is so general and encompass so many processes that I do not believe it is useful in the AOP framework. Perhaps the authors could discriminate two different kinds of signalling to help organize better the complex changes occurring after damaging the brain: one process which is non cell autonomous (immune response for instance), another which is cell autonomous (synaptogenesis defect, cell death, cell senescence).

Reply: We can appreciate the reviewer's point and have clarified further in the text. We believe this KE is sufficiently specific, measurable and essential for advancing the stages of numerous cognitive diseases in the way we have described it, particularly with the additional justifications. In our AOP, we have explicitly highlighted the involvement of critical signaling pathways such as the cAMP-PKA (cyclic adenosine monophosphate-protein kinase A) pathway, the MAPK (mitogen-activated protein kinase) pathway, and the PI3K-Akt (phosphoinositide 3-kinase-protein kinase B) pathway. These pathways are well-documented in the literature for their roles in synaptic plasticity, neuronal survival, and memory formation. Additionally, inhibitor/knockout-based studies to proteins related to signaling pathways show how signaling is important particularly for neuron differentiation and alterations of these pathways, leads to decreased neuron differentiation and downstream cognitive effects (e.g. Zhang et al., 2018).

Also signaling pathways are easily measurable using ELISA and other methods (listed in measurement section of KE). Since the KE is shared among three other AOPs related to cataracts, bone loss and learning and memory impairment, as per OECD guidelines, the descriptions are intended to be general enough to allow reuse for other AOs. More specific details on signaling pathways relevant to cognitive deficits can be found in the KER description of the KER "signaling pathways leading to learning and memory impairment".

In light of the reviewers comment we have now expanded the KE description to highlight autonomous and non-autonomous signaling in the context of dysregulation and adverse effects. The reviewer can refer to page 45 of the snapshot.

Comment: The authors propose the Adverse Outcome as impaired learning and memory. If learning and memory are closely related concepts, the complexity of these functions and diversity of the brain area they mobilise make the AO of this AOP very general. For instance what are the assays used to assess and discriminate these two functions? Are they the same? Is it possible that memory is impacted but not learning (and vice versa?)? I would like the

authors to discuss this point on defining a single AO, while the complexity of cognitive function related to memory and learning is huge. This is for me a strong limitation of this AOP, as it is very difficult to claim that learning and memory are identical processes. I also would like to point that alteration of one brain area can have impact on others, which further blur the notion proposed by the authors that memory and leaning are supported by identical brain structure.

Reply: We note, as per OECD guidelines, we must reuse existing KEs, KERs and AOs already in the AOP knowledgebase if they are appropriate and endorsed. In this case, the learning and memory KE is reused and is part of an endorsed AOP relevant to chemical stressors.

The processes of learning and memory are intricately linked through shared mechanisms involving neuronal plasticity, neurotransmitters, structural changes, and the activation of specific brain regions (Toricelli et al., 2021). Therefore, grouping them together is appropriate and furthermore the processes can be delineated at the measurement level. As described in the KE description, impaired learning refers to the reduced ability to create new associative or non-associative relationships, whereas impaired memory consists of decreased ability to establish sensory, short-term or long-term memories. Both aspects can arise from changes in neuronal architecture as a function of altered synaptic activity, necrosis, demyelination, neurogenesis, neurodegeneration, and dendrite morphology. Although many of the studies that we included in the AOP measure neural remodeling in the hippocampus, we did not limit our search to certain brain regions and our goal is not to suggest that these changes are occurring in one anatomical location. We reported the locations of the studies that were included but more evidence is needed to identify how radiation may impact different areas of the brain. There are various tests that can be used to measure learning and memory independently. These are described in the measurement section of the KE description. We are not trying to suggest that learning and memory are similar processes, in fact, there are multiple ways to test different aspects of learning and memory processes and we included many of these endpoints in the AOP. As discussed in the limitations section, there is more work to be done that translates what these different tests (y-maze, morris water maze etc.) are actually measuring, how slight protocol differences between labs may highlight different aspects of learning and memory processes and how assays in certain models (mouse, rat, etc.) may translate to other species. Answering these questions is not the goal of the current AOP, rather the goal is to consolidate this information to identify research gaps and inform future work.

Indeed, in terms of data to support independent aspects of the AO, studies that we retrieved had measurements representative of both learning and memory.

We have revised the AOP report on page 4 and 5 to indicate that learning and memory are intricately linked as follows:

"Learning and memory are essential cognitive functions and interconnected through common neural networks, synaptic plasticity, neurotransmitters and the interactions between brain regions (Toricelli et al., 2021)."

"Although they are two independent cognitive outcomes, the processes of learning and memory are intimately connected through shared mechanisms (Toricelli et al., 2021)."

Comment: page 3 line 53: "However, data is currently lacking to utilize this approach to estimate risks to the CNS (Nelson et al., 2016; Miller et al., 2022)". The Miller reference is a review on the effects in the CNS but do not describe risks. I do not manage to find the Nelson paper, please provide a doi.

Reply: The review paper by Miller et al., 2022 does present data on biological impact of ionizing radiation on CNS and associated risks observed in dementia and cerebrovascular diseases. The Miller paper also describes risks documented by the Nelson et al., 2016 report. Nelson et al., 2016 is a NASA technical report, reviewed by the NASA Space Radiation Standing Review Panel (https://ntrs.nasa.gov/citations/20160004368). The information on DOI has been added to the AOP report.

Comment: page 4 line 8: "The existing animal and cellular data suggest significant adverse effects of space-relevant charged particles at low (<0.1 Gy) and high (4 Gy) ". Please provide a reference here about these doses.

Reply: We have added a reference to support our statement, Cekanaviciute et al. (2018) review paper. In this paper the authors describe studies and state that doses ranging from 0.05 - 4 Gy have adverse cognitive effects. We took this opportunity to change "<0.1 Gy" to "0.05 Gy". This has been revised in the AOP report page 4.

Comment: Page 6 of the article. The authors make a very nice introduction on the cognitive effects caused by ionizing Radiation (IR) in human. They clearly demonstrate that data are missing at low and moderate dose compared to high dose (as it is very often the case). However I find it difficult to know if the proposed AOP, is applicable to all dose and dose rates, or only to high dose (1 Gy and above). This is especially important for the dose dependent effects related for instance to ROS, for which little evidence exist at dose < 1Gy.

Reply: We agree that it is important to be clear on these details. Figure 5 within the AOP report summarizes the dose ranges used to support the AOP. Minimal studies examine low dose effects, and most studies assess effects at moderate to high doses. Exposure parameter information is also provided in the empirical evidence section and associated tables across each KER that is directly linked to the MIE.

In light of this comment, we now clarify the point that some KERs may be informed by different exposure parameters as follows,

AOP report page 9:

"However, it is important to note that not all types of stressors support each KER and the AOP is not stressor or exposure parameter specific."

Overall assessment section of snapshot on page 4:

"Note that not all types of stressors and associated exposure parameters support each KER."

Comment: The authors cite different references provided page 11 line 51 ("Summary of Scientific evidence) of the article that describe ROS or RON increase. By looking at the different articles cited, one citation provide evidence that ROS can be increased at dose < 1 Gy : Baluchamy et al., 2012. The Baulch et al., 2015 and the Giedzinski et al., 2005 papers provide data showing increase in ROS at dose > 1Gy. The article De Jager, T. L., A. E. Cockrell, S. S. Du Plessis (2017) is on UV, for which I have difficulties to accept as an evidence for an AOP related to IR and space travel. The article from Rehman et al., 2016 does not show effects related to dose in Gy, and to my point of view can not be used to support ROS increase at dose < 1Gy. The citation Tahimic & Globus, 2017, does not provide support on the dose used to increase ROS production. The citation Wang et al., 2019 is a review on Heart diseases and does not provide much information on ROS related to the dose. And finally, the De Jager, T. L., A. E. Cockrell, S. S. Du Plessis (2017) is cited twice. At the end, the support that ROS increase is observed below 1 Gy is only supported by the Baluchamy et al., 2012.

Reply: Thank you for this important comment. Please note that several KERs at the macromolecular level are shared with other AOs. The KER "deposition of energy" to oxidative stress" is shared with AOPs leading to cataracts, vascular remodeling, bone loss and learning and memory impairment. Since each KER is an independent unit from the rest of the AOP, the data used to support it can be drawn from any types of stressors, cell types (as it is a macromolecular event) and exposure parameters (dose, dose-rate, radiation quality). AOPs are driven by biological perturbation and not the stressor parameters. It is important to draw from varied data types from different sources to support the KER, as this then validates the importance of the KER. The KERs are viewed independently, but the overall AOP assessment points to the features that are specific to the domain of this AOP.

Also, we specifically do not make any statements about the dose applicability of the AOP.

In light of this reviewer's important concern and to clarify for the reader, we have revised the text to highlight that data supporting the AOP is not specific to any exposure parameter.

The following has been added to the overall assessment and AOP report:

Overall Assessment section of snapshot on page 5:

"The AOP is qualitative in nature and not intended to be specific to any particular exposure parameter."

AOP Report page 9:

"However, it is important to note that not all types of stressors support each KER and the AOP is not stressor or exposure parameter specific…."

Comment: The same in the wikiAOP webpage. They propose an applicability domain to low and High LET and at 0.1 to 1Gy. But there is very little evidence that oxidative stress is similarly induced at 0.1 and above 1 Gy. Indeed the authors state in the empirical support (on the Wiki AOP webpage) "irradiated with protons at 1, 2, 5 and 10 Gy showed a dose-dependent increase in ROS levels (Giedzinski et al., 2005)."

Reply: The study by Giedziniski et al., is only one example, the complete list of studies can be found in the KER of deposition of energy leading to oxidative stress. Within that KER there are other studies that are relevant to low doses (<0.1 Gy) such as Tseng et al, 2014; Veeraraghan et al., 2011; Baluchamy et al., 2012. Nonetheless, we do not make a claim that the AOP domain of applicability is 0.1-1Gy (See domain of applicability in overall assessment). The intent of stating dose ranges in the description section of overall assessment was to provide readers the information on stressors used to support the AOP. However, we recognize this may be misleading.

In light of this comment, we have added a statement to clarify that description is for the entire AOP, some KERs may be relevant to lower doses, but other KERs to higher doses.

Overall assessment section in snapshot on page 5:

"The AOP is qualitative in nature and not intended to be specific to any particular exposure parameter."

Comment: In the wiki AOP page ("Key Event Relationship Description) they cite the Du Plessis (2017) on UV, which is for me of low relevance in this AOP as it is not a type of radiation that can alter cognition or have neuronal effects. The Karimi et al., 2017 paper is describing effects on lens at 15Gy. In overall, to my point of view the authors, should clarified throughout the paper (and in the KER) how oxidative stress is increased at dose below 1 Gy, as evidence are currently relatively weak.

Reply: As described above, AOPs are stressor agnostic; here we are looking at the biological relationship between the two KEs. Non-ionizing radiation stressors also deposits energy and initiates oxidative stress. Therefore, UV studies are relevant to include, as some studies support the Bradford Hill criteria.

With regard to the comment on oxidative stress occurring below 1 Gy, we agree, a very limited number of studies show effects at less than 0.1 Gy and this is highlighted as either an uncertainty or can be inferred from Figure 5 of the AOP report which summarizes the dose ranges used to support the AOP, the dose-ranges are also listed within each Table provided in the KERs. We also now include a statement that the AOP is not applicable to any specific exposure parameter on page 9 of the AOP report.

Comment: Page 9 line 40. The authors should clearly precise that they focused on neurogenesis in the hippocampus. If not I do not know to which type of neurogenesis, they are refering to.

Reply: Agreed, the following sentence has been revised in the AOP report page 10:

"Neural remodeling includes changes in the morphological properties of neurons as well as altered functional properties such as impaired neurogenesis in the hippocampus. Although neural remodeling is a typically beneficial and ongoing process that enables the brain to adapt, certain stressors such as the space environment can lead to maladaptation, potentially resulting in cognitive deficits despite the brains continued efforts to adjust. Neural remodeling has an adjacent connection to impaired learning and memory [KE#341 in the AOP-Wiki]

whereas both deposition of energy and increased pro-inflammatory mediators have non-adjacent connections to impaired learning and memory."

Comment: page 12 line 26: "and then induce neural remodeling through apoptosis". This is for me an overstatement. Apoptosis is not a process involved in neural remodeling in adult brain after injury. Apoptosis can lead to neuronal death, and consequently synaptic plasticity can compensate for this loss. Or perhaps neurogenesis in very precise location like the hippocampus. In the naive adult hippocampus, new born neurons can undergo apoptosis (see for instance 10.1016/j.stem.2010.08.014), but this is not a process that drive neural remodeling itself in an injured brain. Neural remodeling is a rather consequence of apoptosis.

Reply: Thank you, it has been revised on page 14 of the AOP report as follows:

"Oxidative stress may subsequently lead to neural remodeling through three pathways: (1) by oxidizing DNA bases, which create nicks on the strand and leads to DNA strand breaks (Cannan et al., 2016; Fong 2016) which if persistent can induce neuronal apoptosis disrupting normal neuronal function (Abner & McKinnon, 2004; Desai et al., 2022; Madabhushi, Pan & Tsai, 2014; Michaelidesová et al., 2019; Wang et al., 2017; Zhu et al., 2019); (2) by activation of tissue resident cells in the brain such as astrocytes and microglial cells which lead to increased pro-inflammatory mediators downstream and (3) by inducing changes in multiple signaling pathways, including mitogen-activated protein kinase (MAPK) signaling, phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) signaling, senescence signaling, and apoptotic signaling (Hladik & Tapio, 2016; Simpson & Oliver, 2020)."

Comment: I also find the term neural too vague here, as it can refer to both microglia, astrocyte and neurons.

Reply: Agree, we have revised to say neuronal function

Comment: page 13 line 83: The cited reference provide evidence for increased ROS and proinflammatory signals in glia not in neurons.

Reply: Thank you for pointing this out. The sentence has been revised on page 15 of the AOP report:

"Activation of various pathways including the NF-B transcription factor pathway, the MAPK-AP-1 signaling pathway, and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway could lead to production of pro-inflammatory mediators (Chen et al., 2018; Vezzani & Viviani, 2015). Therefore, pro-inflammatory mediators become abundant in cells of the nervous system such as microglia (Simpson & Oliver, 2020)."

Comment: page 13 line 39 and below. The authors propose a KER as moderate between pro-inflammatory signals and neural remodeling. This term 'neural remodeling' is not supported by the provided literature. Indeed the authors provide information in this section on neuronal cells and their progenitors (for instance neurogenesis, and neuronal differentiation). The term neuronal remodeling usually refers to dendrites outgrowth and synaptic activity, not neurogenesis and neuronal differentiation. The term neural remodeling is encompassing both neuronal and glial cells, but microglia activation and effects of these cytokines is not a neural

remodeling process. The term neural remodeling can be used to describe the process of brain regeneration, but it is not adequately used here to describe how ROS increased in the glial cells can affect neurons and their progenitors.

Reply: We understand the reviewers concern**,** but essentially what we are saying is that an environment of inflammation in CNS can influence neuronal properties (electrophysiological properties in brain), morphological changes in dendrites/synapses, impacts on neurogenesis (e.g.. decreased proliferation and differentiation in progenitor cells, inhibited neural stem cell differentiation), and reduced neuron production.

Papers to support this claim are: Mousa A, Bakhiet M. (2013); Jenrow KA, Brown SL, (2013);Fan LW, Pang Y. (2017) ;Wong WT, Wang M, Li W, et al. (2004) ; Tang Y, Le W. (2017) Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol. 54(3):1770-1778; Cekanaviciute E, Buckwalter MS. (2016) ; Shi Y, Chanana V, Watters JJ, Ferrazzano P, Sun D. (2017); Zonis S, Ljubimov VA (2015)

We also highlight within the KER of altered signaling to neural remodeling that ROS accumulated in glial cells can impact nearby neurons, altering their communication. Therefore, there is strong interconnectivity of glial cells and neurons and (Kim et al., 2020; Linne et al., 2022).

Kim, Y. S., Choi, J., & Yoon, B. E. (2020). Neuron-Glia Interactions in Neurodevelopmental Disorders. Cells, 9(10), 2176. https://doi.org/10.3390/cells9102176

Linne, M. L., Aćimović, J., Saudargiene, A., & Manninen, T. (2022). Neuron-Glia Interactions and Brain Circuits. Advances in experimental medicine and biology, 1359, 87–103. https://doi.org/10.1007/978-3-030-89439-9_4

Comment: The same is true in the next paragraph from page 14 line 36 to page 15 line 22 of the article. The authors describe the effects of inflammatory signals produce by microglia activation on neurons, dendrites, synpases connexion and neuronal progenitors (including effects on neurogenesis). This process is not a neural remodeling effects. It is a neuronal effects caused by inflammation in reaction to injury.

Reply: We believe based on the empirical evidence collected that the neuronal effects from inflammatory signals can lead to decreased proliferation or differentiation in progenitor cells, inhibit neural stem cell differentiation and decrease neurogenesis, which as described in the general comment section above can be a component of maladaptive neural remodeling.

Comment: I also question the authors about the evidence of inflammatory signals at dose < 1Gy. I do not find strong evidence that such process occurs at low dose.

Reply: We agree there is not strong evidence for this. We are not sure why the reviewer suggests that we claim there is high level of data to support that inflammation to remodeling happening at <1 Gy. See our responses to previous comments above.

Comment: Page 15 line 28: “Neural remodeling refers to alterations through changes to neurogenesis, neurodegeneration, neuronal excitability and synaptic plasticity, demyelination and dendritic spine density”. I disagree with the usage of this term neural remodelling. The

authors provide evidences on neurons and their progenitors and cite the Bálentová & Adamkov, 2020 for effects on astrocyte and oligodendrocytes. I think the link between microglia activation and neuronal effects is clear. It is also clear that IR can induce apoptosis of neuronal cells and astroglial cells. But there is no evidence to my point of view that neural remodeling is a cause that lead to impaired learning and memory. Neuronal death, astroglial cells death and induction of inflammation are causing neural damages that lead to the AO. But using the term neural remodeling as a KE that to the AO is not adequate, as neural remodeling is a process that is defined as the mobilization of neuronal and astroglial cells in reaction to damaged structure in order to repair them. I thus disagree with the definition provided by the authors that neural remodeling refers to "alterations".

Reply: Maladaptive neural remodeling can encompass processes related to dendrite outgrowth and synaptic activity, neurogenesis, and neuronal differentiation as described in the general comment section (reviewed in Cekanaviciute et al., 2018; Hladik & Tapio, 2016; Kiffer et al., 2019b; Makale et al., 2017). Within our AOP neuroinflammation as highlighted by the reviewer is represented by the KER of "tissue resident cell activation to proinflammatory mediators", within this KER, we provide specific examples of how pro-inflammatory mediators lead to neuroinflammation which in turn can alter the structure or function of neural cells which can lead to maladaptive neural remodeling and these have downstream consequences to learning and memory processes.

The following is detailed in the report and KER description: Studies have reported changes in the physical and electrophysiological properties of neurons in response to increased cytokine expression, both in whole-brain samples and specific brain regions like the hippocampus or dentate gyrus (Jenrow et al., 2013; Fan and Pang, 2017; Wong et al., 2004). IL-1β, TNF-α, and IL-6 are highlighted as cytokines that cause morphological changes in dendrites and synapses (Tang et al., 2017; Cekanaviciute et al., 2018; Shi et al., 2017). There are also studies that highlight negative impact of proinflammatory mediators on neurogenesis, including decreased proliferation and differentiation in progenitor cells, inhibited neural stem cell differentiation, and reduced neurogenesis (Zonis et al., 2015; Wong et al., 2004; Tang et al., 2017). IL-6 is shown to affect neurogenesis through various mechanisms, including stimulation of the hypothalamic-pituitary-adrenal axis, leading to increased circulating glucocorticoids that inhibit cell proliferation and neurogenesis in the dentate gyrus (Turnbull and Rivier, 1999; Gould et al., 1992; Cameron and Gould, 1994). TNF-α is reported to affect neuronal fate by interacting with its receptor, TNFR1, expressed on neural stem cells. TNFR1-mediated signaling is suggested to inhibit growth, resulting in a reduction in neuron production (Chen and Palmer, 2013).

While a clear mechanistic relationship is not fully established, the evidence provided emphasizes the widely accepted understanding that proinflammatory mediators can indeed alter the structure and function of neurons (Mousa and Bakhiet, 2013).

Comment: page 16 line 34:' although it involves alterations in the neural circuits that regulate these processes'. To which processes the authors are referring to? The phrasing is strange to me. It can be understood as ' alteration in neural circuits regulate inflammation and impair learning'.

Reply: Thank-you for pointing out this unclear text. On page 19 of the AOP report, we revised the sentence to read:

"Although a clear mechanism has not yet been elucidated due to the complexity of inflammatory signaling, sufficient evidence shows that these inflammatory markers are involved in changes to neural circuits that regulate learning and memory processes"

Comment: page 17 line 8: "that examined the effects of IR on the CNS, the doses ranged from 1 cGy to 10 Gy from". The evidence at dose < 1Gy are very week for both ROS production and induction of inflammation. The direct impact from IR to impaired learning and memory is stronger and more convincing as proposed page 16 line 25, the cited references (Cekanaviciute et al., 2018; Kiffer et al., 2019b; NCRP, 2016; Pasqual et al., 2021. But I disagree with the statement 'along with changes in antioxidant levels' page 17 line 15, as these papers used page 16 line 25 are not providing any data on ROS increase. If papers that link IR at < 1Gy to ROS increase and the learning and memory deficit, they must be cited here.

Reply: We have revised the sentence to remove references specifically to the doses. However, we point out that the sentence is founded on the following data that ranges from doses of 1 cGy to 10Gy and assesses RONS and/or antioxidants (indirect indicator of RONS). Also note the KE of oxidative stress is defined as either radical production increase or the loss of protective mechanisms to mitigate the RONS. Both aspects of RONS generation and mitigation are important markers of oxidative stress. As mentioned before, antioxidants that increase in expression are indicative of the presence of RONS. When antioxidants decrease in expression/activity, this is most likely due to the antioxidant defense mechanisms being overwhelmed.

Mice brain tissue following 2, 10 and 50 Gy whole-body gamma irradiation revealed a dose-dependent change in SOD2 activity (Veeraraghan et al., 2011). Mice brain tissue showed decreased glutathione (GSH) and SOD levels following proton irradiation (Baluchamy et al., 2012). Here, the expression of antioxidants is indicative of RONS.

Markers of oxidative stress have also been consistently observed in brain tissue. Human neural stem cells subjected to 1, 2 or 5 Gy gamma rays showed a dose-dependent increase in RONS production (Acharya et al., 2010). A dose-dependent increase in ROS was observed in rat brains following 1-10 Gy gamma rays (Collins-Underwood et al., 2008). Neural precursor cells exposed to 0-10 Gy of X-irradiation showed increased ROS levels (Giedzinski et al., 2005; Limoli et al., 2004). Mouse brain tissue displayed increased ROS following proton irradiation (Baluchamy et al., 2012; Giedzinski et al., 2005). Neural processor cells expressed linearly increased ROS levels following doses of 56Fe (Limoli et al., 2007). A dose-dependent increase in RONS was also observed after exposure to 1-15 cGy 56Fe irradiation in mouse neural stem/precursor cell (Tseng et al., 2014). Human neural stem cells exposed to 5-100 cGy of various ions demonstrated a dose-dependent increase in RONS (Baulch et al., 2015).

To address this comment, we have revised the sentence (page 20 of the AOP report):

"Generally, these studies found a dose-dependent increase in oxidative stress markers in the brain after exposure to IR, along with some studies showing changes in antioxidant levels."

Comment: page 18 line 17: 'neural remodeling following alteration of signaling pathways (El-Missiry et al., 2018; Chow et al., 2000; Suman et al., 2013; Kumar et al., 2005)'. I disagree with the term neural remodeling. These papers show effects on neuronal and glial compartments, like DNA damages but does not provide evidences of neural remodeling being responsible of the adverse effects.

Reply: Thank you for your comment, the papers presented in the KER description highlight activation of pathways leading to cell apoptosis in the brain. This in turn can trigger inflammatory responses that affect surrounding tissue leading to altered neural plasticity and synaptic function, also, which can impair learning and memory functions. Some studies also show apoptotic signaling leading to changes in hippocampal neuron morphology (e.g., total dendritic branch length, number of terminal tips, soma area, spine density, and filopodia density), which in turn can impact learning and memory processes.

Within the KER empirical evidence section, we highlight studies (El-Missiry et al., 2018; Chow et al., 2000; Suman et al., 2013; Kumar et al., 2005) that show in irradiated animal models the presence of markers of neural remodeling (e.g., dendritic structural changes, and apoptotic activity in brain cells) which are linked to learning and memory impairment as described in the KER of "neural remodeling to learning and memory impairment". Some examples of studies are listed below:

- 4 Gy radiation increased cell signaling apoptotic markers (e.g. p53, cytochrome C, BAX, and caspase-3, caspase-8, and caspase-9). This corresponded to increased apoptosis and necrosis. In addition, 4 Gy resulted in extensive damage to the dentate gyrus (El-Missiry et al., 2018). Damage to parts of the hippocampus can alter neurogenesis which is linked to memory impairment
- Increased p53 signaling led to apoptosis levels in neural cells like oligodendrocytes which can impair function (Chow et al., 2000). Oligodendrocytes are responsible for the protection and insulation of axons; the reduction of such cells could decrease their conductivity and connections and impair brain function
- Altered signaling through p16, p21, p53, BAX, and Bcl-2 protein levels lead to increased apoptosis and decreased cortical thickness following irradiation of mice (Suman et al., 2013). These changes have been linked to a gradual reduction in the brain's normal volume from cell death and a decline in cognitive function
- Confocal microscopy was used to assess hippocampal neuron morphology. Inhibition of PI3KAkt signaling pathway significantly reduced the total dendritic branch length, terminal tip number, and soma size (Kumar et al., 2005). Changes in neuron morphology can modulate synaptic strength and influence synaptic plasticity which influences learning and memory. Reduced dendrite size and number would impair or limit brain function.

Since KERs are built as independent units from the rest of the AOP, the two KEs are discussed in the context of current evidence to justify their linkage without regard for downstream effects. This is the reason the connection to the adverse effect is not detailed.

Comment: page 19 line 42:' impaired learning and memory is not observed without exposure to stressors/insults.' This is not completely true, since aging and diseases can also lead to the same outcome.

Reply: Thank you for pointing out that we should clarify this point. That sentence is to highlight the essentiality of deposition of energy. Learning and memory impairment is not observed in non-irradiated young wild-type animals. The sentence has been revised to read as follows on page 22 in the AOP report: "Furthermore, studies have reported that energy deposition from different doses of radiation from X-rays, gamma rays, protons and heavy ions leads to impaired learning and memory, but such impairment is not observed in non-irradiated young wild-type animals."

Comment: page 21: 'Modulating Factors'. The authors should cite age as modulator. In the process of aging increased stress and increases DNA damages or ROS contribute to the onset of learning and memory defects. See for instance psychological stress (10.1016/j.dr.2021.100968) or DNA damages and ROS (https://www.nature.com/articles/s41392-022-01251-0 + https://doi.org/10.3390/antiox12030651)

Reply: Agreed, age is cited as a modulator in the AOP as follows: "Age and sex are also modulators of this AOP as older, aged models have lower levels of antioxidants, greater tissue resident cell activation, increased sensitivity to immune signals and inflammation, as well as greater decrements to radiation-related impairments in learning and memory (Liguori et al., 2018; Hanslik et al., 2021; Casciati et al., 2016; Patterson, 2015; Barrientos et al., 2009; Barrientos et al., 2012)." Note the latter two papers in your comment are not within the dates of our scoping review as they were published after 2021. We have added the Collett et al. paper and the following sentence in the AOP report (page 25), and in the KER of Deposition of Energy leads to learning and memory impairment (page 176 of snapshot):

"Psychological stress related to perceived risk of radiation exposure can also impact learning and memory (Collett et al., 2020)."

Comment: page 22 line 42: "human-specific genes important for learning and memory such as Kallikrein-related peptidase 8". It is not only novel gene expression that drive differences between human brain and other species. The authors should also cite other differences between primate and other mammalian species, such as the presence of oSVZ progenitor that drive much of the brain size differences with rodent for instance (https://pubmed.ncbi.nlm.nih.gov/25695268/)

Reply: Thank you for this suggestion. The reference (Dehay et al., 2015) has been added on page 26 of the AOP report.

Comment: page23 line 83 : "The KE of neuronal remodeling has a wide range of endpoints. Neural remodeling encompass ». Neuronal remodeling and neural remodeling describe processes on different set of cell types. The authors should clarify throughout the manuscript this difference between "neural" and "neuronal". I also do not agree with the proposition that neural remodeling is a KE, as proposed by the authors. The authors state 'Neural remodeling

encompasses changes to the physical and/or electrophysiological properties of neurons' and then cite 'demyelination, neurodegeneration, levels of neurogenesis, synapse formation/remodeling, synaptic activity and dendritic complexity'. The authors describe process that perturb neurogenesis, plasticity, myelination and neuronal electrophysiology. These processes can not be put together as "neural remodeling" which is rather used in to describe extensive remodeling of brain connectivity and regions either during developing or during injury like stroke. The term neural include both glia oligodendrocyte and neurons. The authors describe a series of event from inflammatory signal in microglia to neuron and astrocyte apoptosis and decreased neurogenesis. The process of neurite plasticity, synaptic outgrowth and reshaping brain connectivity are the processes to compensate for these loss, and not a KE that lead to the outcome. The term remodeling is thus not adequate to describe a detriment.

Reply: We acknowledge the reviewers concern on the broadness of the neural remodeling KE. In the inconsistency section of the AOP report we do highlight that our definition of neural remodeling is broad.

While extensive remodeling is often associated with developmental processes or recovery after injury, the term can also be applied to describe potentially maladaptive changes resulting from radiation effects. Ionizing radiation, can affect neuronal and glial cells, leading to changes in synaptic connections, cellular morphology, and overall circuitry through neural inflammation. All of these have been connected to reduced cognitive abilities as assessed in studies that use animal maze tests. A number of different cell types can be involved in cognitive decline and lead to downstream functional and structural changes to neuronal cells. For the AOP we needed to have a tissue level event and the empirical data did not direct us to a specific aspect and a decision was made to collectively refer to these endpoints as "neural remodeling". In the context of the space environment observed changes in neural cells have been shown to lead to downstream cognitive detriments.

Comment: Page 24 line 8: The authors state that ROS production and inflammation are barely observed at dose < 1Gy. The part the AOP with indirect KE leading to the AO, is thus not that simple in the domain of the low dose, despite clinical observation at < 1Gy in human. This is an interesting point, and I would like that this observation that KE ROS and inflammation are mostly observed at dose of 1Gy and above appear clearly in the different parts of the paper and on Wiki AOP, as for instance in the applicability domain as well as in the KE and KER descriptions.

Reply: We agree. This clarification has been added within the uncertainty section of the overall assessment, AOP report and the following KERs: MIE to oxidative stress, oxidative stress to tissue resident cell activation; and tissue resident cell activation to proinflammatory mediators.

Within uncertainty section of the mentioned KERs above the following is stated:

"Limited data is available to support an understanding of this relationship at low doses (<0.1 Gy)"

Uncertainty section of Overall assessment and AOP report (Page 28 of AOP report, page 8 of Overall Assessment section of the snapshot):

"Limited data is available to support an understanding of oxidative stress and pro-inflammatory mediators at low doses < 0.1 Gy."

Comment: KE and KER for resident cell activation and altered signaling pathways are separated in the proposed AOP. This mean that the authors propose that resident cell activation, that contribute to inflammation signals, is distinct from the KE altered signaling pathway. But in the overall Assessment (wikiAOP part Biological Plausibility), it is not clear which pathway belong to which KE as the authors describe" pro-inflammatory mediators and altered signaling pathways can lead to neural remodeling" including proinflammatory signals, senecesnce and apoptosis . Indeed the authors first describe inflammatory cytokines can affect neural remodeling and then state that "these cytokines act on different receptors to initiate several signaling pathways to induce neuronal degeneration, apoptosis or to propagate further pro-inflammatory responses". They thus propose that proinflammatory signals are key to deregulated pathways like neuronal degeneration, apoptosis responsible for neural remodeling. I am thus not sure that the position of the KE 2066 on altered signaling pathways is appropriate. Time concordance of the different KE 2066, 1492 and 1493 is thus not clear.

Reply: The AOP flow diagram is organized based on biological levels (macromolecular, cell, tissue, organism) not on time the KE is presented. Signaling pathways and proinflammatory mediators act as feedback loops acting under similar timeframes. Under the time concordance section of the AOP (page 7 of snapshot) we indicate: "For tissue resident cell activation and increase in pro-inflammatory mediators, studies generally show that these events occur at a similar time frame (Parihar et al., 2018; Liu et al., 2010; Dong et al., 2015; Lee et al., 2010; Zhou et al., 2017). The alteration of signaling pathways is a molecular-level KE like oxidative stress, and both can occur concurrently (Xu et al., 2019), although increased ROS levels can be initiated significantly before altered signaling pathways (Suman et al., 2013)."

Comment: I also do not think that altered signaling pathway is a real KE. This is too vague to be measured. The authors pinpoint in the text (both in the wikiAOP and in the article) to several pathways that are pertinent. The KE 2066 must be better defined to describe a proper pathway related to specific cell of tissular effects. This link between KE 2066 and KE 2098 is too broad.

Reply: Altered signaling is an important KE in the AOP, it is well recognized that when signaling molecules is persistent or insufficient, it can culminate in many diseases. Furthermore, the KE is shared among many AOPs.

For the purpose of this AOP, there are a few pathways related to apoptosis that have been consistently highlighted as being involved in cognitive deficits. The reviewer is directed to Figure 2 of the AOP report and within the appropriate KER description (page 126 of snapshot) where it indicates: "Neural remodeling can be induced by changes in multiple signaling pathways, including MAPK signaling, PI3K/Akt signaling, senescence signaling, and apoptotic signaling".

We further describe how these pathways are involved in the homeostatic regulation of neuron numbers, morphology, proliferation, differentiation, and synaptic activity. To narrow the KEs to one specific pathway will require more research, this is highlighted as knowledge gap in Table 4 of the AOP report. What is discussed within the KER description accurately reflects our current state of knowledge on signaling pathways.

Comment: In the wikiAOP I think the Applicability domain is justified only for rat, mice, human, dog, but not for the other species.

Reply: Since this KE is shared among other AOPs, pigs, cows and rabbits are also relevant, and therefore we have kept it in the applicability domain.

REVIEWER #2

KEY EVENTS

Key Event 1 (Molecular Initiating Event, MIE): 1686, Deposition of Energy
Note: Shared KE, Previously reviewed in AOP 272
Comment: The depiction of energy deposition as the Molecular Initiating Event (MIE) is well-justified and have been discussed and agreed upon at several dedicated forums. However, it would be useful to clarify the specific mechanisms by which energy deposition initiates the cascade leading to oxidative stress. Attention to the types and sources of energy that are most relevant to this AOP would enhance its applicability. Excitation of molecules upon irradiation is also a deposition of energy - is it relevant to the KE? What % of deposited energy, e.g., upon gamma-irradiation, is excitation vs. ionization? And why excitation energy is not listed in Table 1 for the MIE?

Reply: We agree. Please note that the KE description is intended to provide sufficient details to understand what it is and how it is measured. Details on how energy deposition leads to oxidative stress can be found within the KER of "deposition of energy leading to oxidative stress". Specific details on sources, energy, types that support the AOP can be found in the empirical evidence section of each KER. Mention of excitation has been added to the KE description.

To address this comment, the following revision has been made on page 31 of the snapshot:

"Ionizing radiation can cause the ejection of electrons from atoms and molecules, thereby resulting in their ionization and the breakage of chemical bonds. The excitation of molecules can also occur without ionization. These events are stochastic and unpredictable. The energy of these subatomic particles or electromagnetic waves ranges from 124 KeV to 5.4 MeV and is dependent on the source and type of radiation (Zyla et al., 2020)."

Key Event 2: 1392, Oxidative Stress
Note: Shared KE, Previously reviewed in AOP 17 and 220

Comment: This KE is fundamental in the AOP and is well-supported by empirical evidence. However, the current definition of KE 1392 is not sufficiently specific (even its description in Table 1 gives two very broad bullets, and not specific molecules or families of molecules involved). Although all specific molecules involved in the context of ionizing radiation are described in the texts and its various parts (IR), the KE seems to be too broad. Its ambiguity is also highlighted by opposite possible interpretations of down- and up-regulation of antioxidant enzymes. Within the context of this AOP it is understood that down-regulation only is a marker of oxidative stress. However, alone this readout is insufficient / indirect evidence for the presence of the oxidative stress. Besides, many authors interpret up-regulation of the anti-oxidant enzymes as oxidative stress, which is opposite to the logic used in the AOP 483.

Reply: We note that this KE is used in many other AOPs and is part of an "endorsed" AOP. Also note that measurements and specific molecules are listed within the section labeled "sources of ROS production".

Within the AOP report, the table on measurements is intended to provide the key bullets of the dominant measurements used to support the AOP it is now expanded to include more endpoints. Detailed information can be found within the AOP Wiki snapshot and listed references.

In terms of opposite effects related to antioxidants following radiation exposure, the authors have included the following in the inconsistency section of MIE to oxidative stress KER as seen on page 83 of the snapshot:

"Antioxidants that increase in expression are indicative of the presence of RONS. When antioxidants decrease in expression/activity, this is most likely due to the overwhelming of the antioxidant defense mechanisms."

Key Event 3: 2066, Altered Signaling Pathways
Comment: The name of the KE should be consistent throughout the manuscript (e.g. it is called differently on the diagram and Table 1). The alteration of signaling pathways is a critical step in the AOP, linking initial oxidative stress to cellular responses. However, KE 2066 lacks specificity in describing altered signaling pathways. The human cellular system contains vast and complex network of signaling pathways in human cells, each with distinct roles and responses to external stimuli. It's accurate to state that all cellular functions are regulated by changes in signaling pathways. To enhance the precision and relevance of this KE, it is imperative to delineate the specific signaling pathways that are critically affected by IR and elucidate how these alterations drive the subsequent key event, KE 2098 - Increase in Neural Remodeling. While the manuscript and AOP address certain pathways with supporting evidence and their connection to neural remodeling, this crucial information is not readily apparent in the AOP or the KE description, potentially diminishing the AOP's utility by allowing critical details to be overlooked.

Reply: Thank you for noting the inconsistency in the KE name. This has now been rectified in the Table. In terms of the comment on the lack of details presented in KE descriptions, we have now added more detail related to how dysregulation of signaling can lead to downstream detriments. In addition, thethe relevant KERs describe more details of specific pathways,

empirical support, essentiality, modulators etc. However, in light of the reviewer's comments we have revised the KE description to be more specific regarding some pathways that when dysregulated are associated with disease processes. Please refer to page 45 of snapshot.

Comment: The description of this KE in Table 1 is overly broad and should focus on identifying key specific pathways instead of providing a generic definition of what a signaling pathway entails. Additionally, the 'how to measure' column in Table 1 lacks detailed guidance; the current information is insufficient for users aiming to measure the relevant molecules or changes experimentally. It would be more beneficial to include references to specific assays, kits, and methods associated with the molecules detailed in Fig2, thereby offering clear and actionable instructions for experimental measurement.

Reply: We have revised Table 1 in the AOP report. The AOP report and tables are intended to be simplified versions of the content in the AOP Wiki (snapshot). The KE in the AOP Wiki provides a table of measurements and Figure 2 of the AOP report summarizes the specific measurements that informed the empirical evidence. In the legend of the table, we direct readers to the AOP Wiki and Figure 2 for more details.

Key Event 4: 1492, Tissue resident cell activation
Note: Shared KE, Previously reviewed in AOP 17 and 38
Comment: Tissue resident cell activation is indeed a pivotal event, particularly in the context of neuroinflammation. However, the term 'tissue resident cells' covers a broad range of cell types in different tissues. Probably, it would be beneficial to consider brain specific title of this KE abd to discuss the types of cells involved and their activation mechanisms in the context of IR exposure and the AO in question.

Reply: AOP developers are strongly encouraged to reuse existing KE in AOP Knowledgebase and consider naming KEs so other AOPs can also be built from them. This is a fundamental principle of AOP development that we are required to adhere to. It is the network of AOPs that is meant to be the unit of application (requiring shared KEs and KERs). The domain of application of the overall AOP is as narrow as the narrowest domain of application of the KEs and KERs. Thus, although many of the KEs and KERs are broad, the overall AOP is only relevant to the cell types associated with deficits in learning and memory. This KE already existed in the AOP Wiki in an endorsed AOP and was thus reused; it is applicable to a wide variety of diseases and therefore brain specific title is not appropriate.

The reviewer is directed to the relevant KERs (e.g., tissue resident cell activation leads to proinflammatory mediators) for Cell/Tissue specific information. Within the KERs, relevant cell types related to the AO are discussed. For example, under biological plausibility we state on page 102 of snapshot: "There is an abundance of studies that explore this relationship using the brain microenvironment, where astrocytes and microglia are the primary tissue-resident cells. After activation, these cells increase in number (whether through proliferation or recruitment), undergo morphological changes and release cytokines".

Additionally, within the AOP report and overall assessment we also highlight the specific tissues the AOP is applicable to as follows on page 5 of the AOP report:

"There are multiple brain areas involved in learning and memory including the hippocampal region, imperative for declarative or episodic memory and the process of long term potentiation, the amygdala, which can process emotional components to memory, the parietal lobe, which is involved in spatial memory, the prefrontal cortex, involved in regulating emotional behaviors, thoughts and actions and the basal ganglia, which may be important for stimulus response associations. These areas do not act independently as multiple brain areas may be involved at any given time depending on the task or stimulus"

Key Event 5: 2097, Increase, Pro-Inflammatory Mediators
Comment: This KE is wrongly given on the AOP diagram as KE 1493.

Reply: Thank you for noting this, we have now revised the figure to KE 2097.

Comment: The increase in pro-inflammatory mediators is a well-established response to tissue damage and stress. Similar to the previous KEs, however, the KE could include more specificity to provide more insights into what mediators or groups of mediators contribute to neural remodeling.

Reply: As noted above**,** the KE descriptions are intended to provide sufficient details to understand what the KE includes and how it is measured. The KEs are reusable and meant to be written in a way not specific to a type of stressor or an AO. This KE is essentially reused. In terms of specificity in the context of neural remodeling, this is described in more detail within the empirical evidence of KER descriptions, this includes tissue and cell type information.

Note in the KE description it indicates: "This event occurs equally in various tissues and does not require tissue-specific descriptions…."

In light of reviewer's comments, we have modified the KE and expanded the description to include how pro-inflammatory mediators can have dual role and dysregulation can lead to adverse effects. The reviewer should refer to the snapshot on page 58-59 for the tracked revisions.

Comment: It is extremely important to use literature that maintains the brain context, as it is well known that immune regulation is highly context-dependent and common pro-inflammatory mediators such as TNF-alpha and IL-6 can execute an anti-inflammatory function (Pro-inflammatory cytokine TNF-alpha as a neuroprotective agent in the brain - PubMed (nih.gov); Anti-inflammatory effects of tumour necrosis factor (TNF)-alpha are mediated via TNF-R2 (p75) in tolerogenic transforming growth factor-beta-treated antigen-presenting cells - PubMed (nih.gov); Defining the Role of Anti- and Pro-inflammatory Outcomes of Interleukin-6 in Mental Health - PubMed (nih.gov); Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke - PubMed (nih.gov)); examples for other molecules identified by the authors as anti-inflammatory factors can be found.
Perhaps, this KE spans both 'cell' and 'tissue' levels. At the cellular level, they are produced by individual cells in response to stimuli, while at the tissue level, their collective action and distribution- at a distance away from the original production and secretion - influence the overall inflammatory response within the brain tissue, impacting its function and health.

Reply: Thank you for that comment, we agree this is important information. However, maintaining tissue relevant details is better discussed within KERs at the cell/tissue level. For example, within KER tissue resident cell activation and pro-inflammatory mediators we provide examples for brain specific cell types involved with the KER.

The authors agree with the comment that "common proinflammatory mediators (TNF-alpha and IL-6) can execute both inflammatory and anti-inflammatory role". This has already been highlighted for IL-6 within the "inconsistency" section of the KER pro-inflammatory mediators leading to neural remodeling and within AOP report.

The following has been added in the AOP report on page 28:

"Inflammatory markers can have both anti-inflammatory and pro-inflammatory role. Factors such as concentration, timing, and the specific microenvironment can influence whether a mediator acts as pro-inflammatory or anti-inflammatory." We now also cite some of the papers shared by the reviewers to support this statement.

The following is in the inconsistency section of the KER on page 112 of the snapshot: "It has also been reported that TNF-α exhibits neuroprotective effects as their transmembrane receptors can influence different signaling pathways (Figiel, 2008; Masli & Turpie, 2009)."

With regard to the reviewer's comment related to collective action of pro-inflammatory mediators, the following has been added in the biological plausibility section of the KER page 109 of snapshot: "It is known that cytokines and their receptors are constitutively expressed by neurons in the central nervous system, and even in normal or pathological states, these cytokines can be produced by individual cells and act on neurons. At the tissue level, the collective action and distribution of the cytokines can influence the overall inflammatory response within the brain tissue, impacting its function (Kishimoto et al., 1994)"

Key Event 6: 2098, Increase, Neural Remodeling
Comment: Neural remodeling as a last step prior to the AO is a crucial KE. Much like with the previous KEs, however, the authors should make an attempt to provide more specificity to this KE definition. The manuscript should focus on detailing the mechanisms of neural remodeling in response to the previous key events. It would be beneficial to discuss how these changes directly lead to the adverse outcome of learning and memory impairment, paying a special attention to physiological characteristics of the key event, e.g., the brain domain specificity. Also, the manuscript should clearly define what is meant by 'neural remodeling' in the context of IR-induced effects. Neural remodeling can encompass a range of processes, from synaptic plasticity and neurogenesis to dendritic pruning and changes in neural circuitry. The manuscript should specify which of these aspects are most relevant to IR exposure and the progression of this AOP. Such specificity will not only enhance the scientific validity of the AOP but also its applicability in predicting and managing IR-induced neurological effects.
The authors should consider changing the level from cell to tissue for this KE. Whereas some of the endpoints listed within this KE are indeed properties of individual cell, the KE, as defined refers t a tissue level process, the process by which the structure of the brain's neural networks is changed. Neural remodeling is a characteristic of both individual neurons and neural circuits

within the brain tissue, reflecting the dynamic nature of the brain's architecture in response to internal and external stimuli.

Reply: We agree and have revised this KE to a tissue level KE in Figures 1 and 2, thank you for the suggestion. In terms of more details specific to IR, the reviewer is directed to the KER description of "neural remodeling leading to learning and memory impairment" where we explain these mechanisms in more detail. As noted, KE descriptions are not intended to be stressor specific. However, in light of the reviewer's comment the KE description of the neural remodeling has been revised to include information specific to neural remodeling in the context of adverse effects that can lead to learning and memory impairment. Refer to snapshot for tracked revisions on page 62-63.

Key Event 7: 1635, Increase, DNA strand breaks
Note: Shared KE, Previously reviewed in AOP 272 and 296
Comment: It is somewhat surprising that even this, one of the first in the AOP Kes, lacks specificity. It is well known that single-strand breaks and double-strand breaks can lead to vastly different consequences and trigger very different signaling pathways. This KE should be revised to address this. The implications of specific DNA strand breaks in neurons and possibly glial cells, in particular, should be highlighted.

Reply: This is a reused KE from an endorsed AOP. KE descriptions do not detail downstream or upstream events in the AOP that are related to the KE as these can be different depending on the AO the KE is linked to. KEs are described in a manner that can be reused by other AOP developers and applied to different AOs. The depth of information presented should be sufficient to understand the measurable endpoints that encompass the KE and what the KE is. For DNA strand breaks, we describe the important types of breaks and some information on how they may be formed. More details on how the interaction is related to radiation are found within KERs directly linked to the MIE of deposition of energy. The implications of DNA strand breaks on neurons are detailed within the relevant KER description of DNA strand break to neural remodeling .

Key Event 8 (Adverse Outcome, AO): 341, Impairment, Learning and memory
Note: Shared KE, Previously reviewed in AOP 12, 13 17, 48, and 54
Comment: As the adverse outcome, the impairment in learning and memory is the culmination of the AOP. The manuscript would benefit from, like with the 'neural remodeling', more attention to specific brain regions where the pathology occurs.

Reply: This is a reused AO from an endorsed AOP. The information presented in the description is sufficient to understand what the KE is and how it is measured. Within the description an overview is provided of what entails learning and memory, how the two work together, the main brain regions involved and what "impaired" refers to. Details on brain regions can also be found in specific KERs linked to neural remodeling and also summarized in AOP report on page 5.

Comment: The description of the AO may also address the potential for reversibility or mitigation of these effects.

Reply: Agree, this information is described within the modulating section of each KER description.

Comment: Another suggestion is to consider whether the AO is a tissue vs. organism level effect. Most of the endpoints (if not all) listed within the AO (Fig 2) are characteristics of an organism, specifically of individuals who have the capacity for conscious recall of facts and events, cognitive capacities, etc. While they involve brain tissue, particularly regions such as the hippocampus and temporal lobes, memory and cognition as concepts refers to the function and capability of the entire organism to encode, store, and retrieve information consciously.+

Reply: Thank you for this comment. In the AOP Wiki it is defined as an organism event, Figures 1 and 2 have been revised to reflect it as an organism level event.

KEY EVENT RELATIONSHIPS

Adjacent KERs

2769, Energy Deposition leads to Oxidative Stress
Note: Shared KER,
Comment: Decreased, as well as increased activities of antioxidant enzymes should not be equated to oxidative stress. Oxidative stress is assessed by direct measurement of the levels of ROS or RNS. If the authors postulate that decreased antioxidant enzymes are markers of oxidative stress (e.g. use of reference Klucinski et al 2008), then the authors may need to explain how studies showing the opposite fit in supporting this KER.

Reply: Agreed. We have now clarified that studies measuring both RONS and antioxidants across a broad dose range will see an increase in antioxidants with the purpose of mitigating this stress. Lower doses produce enough RONS to mitigate the stress but at much higher doses these enzymes are overwhelmed, and the expression or activity of antioxidants declines. This information has now been added to the KER (see below). For example, antioxidant enzyme activity initially increased by a statistically negligible amount from 0-2 Gy and then decreased in a dose-dependent manner from 2-8 Gy (Kook et al., 2015).

Within KER inconsistency section we indicate on page 83 of the snapshot: "Antioxidants that increase in expression are indicative of the presence of RONS. When antioxidants decrease in expression/activity, this is most likely due to the overwhelming of antioxidants."

2832, Energy Deposition leads to Tissue resident cell activation
Comment: This KER ought to be excluded from the AOP due to the absence of any identified biological mechanism that establishes a direct connection between the two KEs. It is through the preceding KEs that the MIE results in tissue resident cell activation.

Reply: This is a non-adjacent KE, we include, where possible any connection of KEs to the MIE, as this type of data can help with strengthen the weight of evidence for the overall AOP through use of the Bradford Hill empirical evidence criteria. Multiple MIE to KEs across the AOP can also help with the quantitative understanding of the AOP (i.e., linking how much energy deposition cause a x% change in downstream events). There is often limited quantitative data between adjacent relationships and non-adjacent relationships are included in AOPs to address this. Furthermore, this KER in particular also describes details on cell types that are activated in the brain and helps address comments below.

2771, Oxidative Stress leads to Altered Signaling
Note: Shared KER
Comment: This KER possesses significant biological plausibility, largely attributable to the expansive interpretation of 'Altered Signaling.' Essentially, any variation in gene expression or post-translational modification of proteins following oxidative stress can serve as corroborative evidence for this KER. Consequently, assessing this KER is challenging without first narrowing down the definition of the Key Event "Altered Signaling." For example, dose and time concordance will greatly depend on what readout is used for altered signaling and those readouts can be early and late type of responses to external stimuli.

Reply: We fully agree that there is significant biological plausibility to justify the qualitative importance of this KER. It is essential to many diseases and applicable to many cell types. Within the KER we describe the predominant specific pathways activated that are relevant in the brain cells. Additionally, since this KER is shared across multiple AOPs and is a macromolecular level event, it is applicable to many cell types – we narrow this domain in our KERs and in our overall AOP. This aspect further guided finding sufficient empirical evidence to justify the importance of the KE.

In terms of the comment related to the type of readout determining the dose and time concordance relationship, we agree. This is the reason the quantitative understanding of the relationship is low. It is clear that a consensus is needed to establish the best test methods and then generate appropriate quantifiable data. In light of this comment, we have added a statement in the "uncertainty and inconsistency" section of the KER on page 110 of the snapshot:

"The assays employed in studies to assess the KEs may lead to variations in the quantitative understanding of observations."

2833, Oxidative Stress leads to Tissue resident cell activation
Comment: For this reviewer, it is unclear how tissue resident cell activation could proceed without the involvement of signaling cascades or pathways as effectors. This perspective stems from the widely accepted understanding of cellular response mechanisms to stimuli, which involve signaling processes. Therefore, it is strongly advised to contemplate the exclusion of this KER from the AOP.

Reply: Thank you for this question. Within the KER description we explain how oxidative stress can lead to tissue resident cell activation. In the brain, free radicals can activate microglial cells and astrocytes. Both microglial cells and astrocytes can change from resting to reactive states, termed gliosis, in response to excess RONS. For example, activated astrocytes can be measured, characterized by hypertrophy (enlargement of cell bodies and processes) and/or an increase in the expression of certain proteins, including glial fibrillary acidic protein (GFAP). In terms of microglia activation, there are various proteins that are upregulated during their activation such as CD68, Iba-1, Mac-1, and ED1. We were able to identify measurable endpoints that represent both KEs (Figure 2) and show a good level of empirical evidence, including that if oxidative stress is removed, neural cells are not activated. Together, this justified the final decision on the inclusion of the KER within the AOP. Furthermore the "altered signalling" pathway KE was focused more on apoptotic signaling and not on ROS.

2834, Tissue resident cell activation leads to Increase, Pro-Inflammatory Mediators
Comment: The link between tissue resident cell activation and pro-inflammatory signaling is well recognized in the scientific community. However, the authors should focus on citing studies that are directly relevant to the experimental models under consideration. It is questionable whether findings from, for instance, mouse kidney (Scharpfenecker et al., 2012) or human monocytic leukemia cell lines (Lodermann et al., 2012), can substantiate this KER, given its specificity to particular tissues and contexts. Therefore, it is advised that the authors

refine the references cited for this KER, a task that would be simplified by redefining the KE as previously suggested.

Reply: As described above, KERs are independent units from the rest of AOP; therefore, studies from any types of cells/tissues can be used to justify the causal linkages of the KERs. The KERs are meant to be reusable for other AOs. It is thus acceptable to include the two references (Scharpfenecker et al., 2012 and Lodermann et al., 2012) in the empirical evidence section of the KER.

2835, Increase, Pro-Inflammatory Mediators leads to Increase, Neural Remodeling
Comment: This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

Reply: As described above, the proinflammatory mediator KE already exists in the AOP Wiki, and is reused. By maintaining a broader scope for neural remodeling, we aim to acknowledge the complexity of the system and avoid overlooking potentially relevant effects that may emerge as more evidence becomes available. The specificity of endpoints used to support the AOP is summarized within the specific KERs and Figure 2 of the AOP report that highlights endpoints that provide basis of empirical evidence.

2836, Increase, Neural Remodeling leads to Impairment, Learning and memory
Comment: This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of Neural Remodeling.

Reply: As described above, the learning and memory KE was reused (already in the AOP Wiki). With regard to neural remodeling, please also see the comment above. Due to uncertainty in exact mechanisms and the broad scope of knowledge in this area, we felt it would be appropriate to have a broader scope for the KE of neural remodeling. It is known to encompass many processes. Keeping a broader scope also helped find relevant empirical evidence. Nonetheless we highlight within the AOP report that the KE of neuronal remodeling has a wide range of endpoints. Neural remodeling encompasses changes to the physical and/or electrophysiological properties of neurons. Several endpoints are usually measured/analyzed for the KE such as demyelination, neurodegeneration, levels of neurogenesis, synapse formation/remodeling, synaptic activity and dendritic complexity (spine number and density). Variations between protocols in different studies are the main source of inconsistency.

2840, Altered Signaling leads to Increase, Neural Remodeling
Comment: This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

Reply: As discussed above. Altered signaling is an essential event, dysregulation of signaling pathways can lead to many disorders. We do provide more specificity within the KER descriptions.

2841, Increase, DNA strand breaks leads to Increase, Neural Remodeling
Comment: To this reviewer, the direct progression from DNA breaks to neural remodeling without intermediate biological events is unclear. Notably, even early neural remodeling

endpoints, such as cell death, necessitate the involvement of various pathways, including DNA repair, cell cycle regulation, and apoptosis, all of which are mediated by signaling cascades. Consequently, it is advised that this KER be reconsidered for inclusion in the AOP due to the necessity of these intermediate steps.

Reply: This is a non-adjacent relationship. The intent of including is to provide additional empirical evidence (studies that measured DNA strand breaks and neural remodeling) to justify the relevance of KEs, linkage to AO and strengthen the weight of evidence.

2811, Oxidative Stress leads to Increase, DNA strand breaks
Note: Shared KER
Comment: This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

Reply: Oxidative stress and DNA strand breaks are well established KEs in the AOP Wiki, and they are integral to many existing AOPs. By maintaining a broader scope, we aim to acknowledge the complexity of the system under investigation and avoid overlooking potentially relevant effects that may emerge as more evidence becomes available. The specificity of the response is summarized within the specific KERs and Figure 2 of the AOP report that highlights endpoints that provide basis of empirical evidence. DNA strand breaks are represented by single strand breaks, complex lesions and double strand breaks. Oxidative stress is represented by the free radicals and antioxidant defense mechanisms. Limiting scope of KEs also makes it difficult to find studies that support Bradford Hill criteria.

1977, Energy Deposition leads to Increase, DNA strand breaks
Note: Shared KER, Previously reviewed in AOP 272
Comment:
No comments

2856, Increase, DNA strand breaks leads to Altered Signaling
Note: Shared KER
Comment: This KER might need modification in light of the earlier recommendations, aiming for a more precise definition of these KEs.

Reply: By maintaining a broader scope, we aim to acknowledge the complexity of the system under investigation and avoid overlooking potentially relevant effects that may emerge as more evidence becomes available. The specificity of the response is summarized within the KER and Figure 2 of the AOP report which highlights specific endpoints that provide the basis of empirical evidence. For example, we highlight that specific pathways related to neural remodeling are altered as a result of DNA strand breaks.

Non-adjacent KERs

2837, Energy Deposition leads to Increase, Neural Remodeling
Comment: It is not clear how this KER should be part of the AOP since the whole purpose of the AOP is to generate a causally and mechanistically linked chain of biological key events; from early to late events, such as these two KEs. Thus, positing a direct linkage between them

appears implausible, suggesting a reevaluation of their inclusion is warranted for coherence with the AOP's foundational principles.

Reply: Non-adjacent KERs are simply used to further explore the quantitative aspect of empirical evidence (Bradford Hill dose, temporal and incidence concordance between KEs) to strengthen the overall weight of evidence of AOP.

2838, Energy Deposition leads to Impairment, Learning and memory
Comment: Same as for KER 2838

Reply: The non-adjacent KERs provide good quantitative evidence that further supports the AOP. For example, each MIE to KEs can be used to better understand the dose-response relationships, as many studies do not measure two adjacent KE.

2839, Increase, Pro-Inflammatory Mediators leads to Impairment, Learning and memory
Comments: Same as for KER 2838

Reply: We have provided detailed rationale for why non-adjacent relationships are included in AOPs above.

OVERALL AOP PAGE

Comment: The "AOP 483 snapshot" document outlines the development of an AOP related to the deposition of energy leading to learning and memory impairment. It details the sequence of key events starting from the MIE of energy deposition, through various biological processes such as oxidative stress, altered signaling pathways, tissue resident cell activation, and increased pro-inflammatory mediators, leading to neural remodeling. The culmination of these events results in the adverse outcome of impaired learning and memory. The document provides a comprehensive overview of each key event, including their biological basis and interconnections within the pathway. The authors carried out a robust review of literature using a modified systematic review approach and should be commended for an outstanding effort. The resulting AOP is a significant advancement in the field. However, given the breadth of the scope (space/cosmic radiation and other radiation types) and the complexity of the multifactorial adverse outcome relevant to behavioral changes, the AOP would benefit from further revision that would address comments and concerns expressed in this review.

Reply: Thank you for positive feedback.

Comment: The generic nature of the key events used by the authors makes it very difficult to access the aspects of the AOP such as empirical support. Indeed, the authors themselves Refer to studies that measure specific markers such as P53 BAX, BCL-2 etc. But insufficient specificity is provided for tissue remodeling or altered signaling pathways: *"Few studies showed incidence concordance where the upstream KE demonstrated a greater change than the downstream KE following a stressor. Not all KERs displayed an incident-concordant relationship, but for those that did, only a small proportion of the empirical evidence supported this relationship. For example, mice exposed to 2 Gy of gamma irradiation showed increases of pro-apoptotic markers p53 and BAX by 8.4- and 2.3-fold, respectively. A 0.6-fold decrease in Bcl-2 (anti-apoptotic marker) was also observed, and gamma rays cause a decrease in cortical thickness*

by 0.9-fold (Suman et al., 2013).” This is an example how the use of generic key events undermines the utility of the AOP concept.

Reply: As described above, we acknowledge the generic nature of the KEs in our AOP. More specificity is provided within the KERs. At present, we believe that a comprehensive approach is essential to capture the multifaceted nature of cognitive impairment. As highlighted in Figure 2 of the AOP report, we aimed to provide transparency regarding the predominant endpoints that informed our AOP. This figure offers specificity in terms of the endpoints that contributed to the development of our pathway (despite the generic name of the KE). Overall, while we understand the importance of specificity, our chosen KEs are broad to allow reuse and reflect the available evidence. Furthermore, AOPs are not static – they can be modified as new evidence emerges.

Comment: The lack of positive and negative feedback loops in the AOP significantly undermines the process' complexity and regulatory intricacies. Positive feedback mechanisms, such as the induction of ROS by pro-inflammatory responses, as well as negative compensatory circuits, such as induction of anti-oxidant enzymes upon altered signaling pathways, are critical for understanding and describing the progression of neurological damage. This oversight simplifies the dynamic and interconnected nature of brain responses, potentially leading to inaccuracies in predicting the severity and progression of radiation-induced cognitive impairments. Incorporating these loops is essential both for scientific accuracy and for enhancing the predictive accuracy of the AOP and applicability (e.g.., guiding effective interventions, risk prediction, etc.).

Reply: We agree. Where data were available, information on feedback loops was provided within each KER. The following KERs include information on feedback loops:

- Oxidative stress leads to altered signaling
- Oxidative stress leads to tissue resident cell activation
- Tissue resident cell leads to increased pro-inflammatory mediators
- Deposition of energy leads to impaired learning and memory
- DNA strand breaks leads to neural remodeling
- Deposition of energy leads to oxidative stress

Comment: The authors should specifically consider a feedback loop from a pro-inflammatory mediators secreted by neural resident cells to alterations in signaling pathways. This would highlight the intricate relationship between inflammation and signaling pathway modulation within the brain.

Reply: We provide details on feedback loops in the KER “Tissue resident cell activation leading to proinflammatory mediators” as seen on page 107 of the snapshot: “It is well-characterized that activated tissue-resident cells can increase expression of pro-inflammatory mediators (Hladik & Tapio, 2016; Lumniczky, Szatmari & Safrany, 2017; Kaur et al., 2019). However, there exists a feedforward loop for this KER as pro-inflammatory mediators can also activate tissue-resident cells within the brain and perpetuate the inflammatory response (Kim & Joh, 2006; Vezzani & Viviani, 2015). Thus, after stimulation by cytokines, chemokines or inflammogens such as from damaged neurons, microglia and astrocytes activate inflammatory signaling pathways, which result in increased expression and/or release of inflammatory mediators such

as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001; Bourgognon & Cavanagh, 2020). Various studies have shown that overexpression of IL-1β in mouse models resulted in the appearance of inflammatory markers including activated glial cells and increased pro-inflammatory cytokine and chemokine mRNAs (Hein et al., 2010; Moore et al., 2009). Additionally, IL-6 plays a role in activating glial cells as mouse models with IL-6 knocked out showed reduced astrocytic population, as well as a reduced ability in activating microglia (Klein et al., 1997). Cytokines and chemokines can also increase the permeability of the blood-brain barrier, further increasing pro-inflammatory mediator levels (Lumniczky, Szatmari & Safrany, 2017)."

Comment: Page 21: Correct "UVC radiation (X-X nm)"

Reply: This is no longer in the overall assessment section of the snapshot.

Comment: Referencing studies on ultraviolet (UV) radiation effects, such as de Jager, Cockrell, and Du Plessis (2017), which explore the impact of UV on antioxidant enzymes, does not seem appropriate. This is because UV radiation primarily affects the skin and does not penetrate deeply enough to directly impact brain tissues or functions.

Reply: In accordance with the OECD AOP guidelines, the AOP approach is designed to be stressor-agnostic. This means that various types of stressors can be utilized to substantiate the causal connectivity within the relationship. UV radiation, being a relevant stressor not only on Earth but also in space environments, aligns with this stressor-agnostic principle. We can use this stressor for the non-brain specific effects to support the upstream relationships in the AOP. This enhances the robustness of our AOP by acknowledging the diverse stressors that contribute to the oxidative stress KER, emphasizing its relevance in different environmental settings, including space. UV radiation can induce oxidative stress, therefore it is biologically plausible for UV to initiate downstream events to the AOP. Data related to UV exposure is particularly relevant to cataracts, which is an AO in our network of four AOs (i.e., cataracts, vascular remodeling and bone loss in addition to learning and memory).

AOP REPORT MANUSCRIPT

Comment: Issue with the generic nature of many key events is exemplified by the KE 1493: pro-inflammatory mediators can exhibit anti-inflammatory effects under certain conditions. This paradoxical role well known and is a part of the complex and dynamic nature of the immune system. The function of pro-inflammatory mediators can be context dependent. Factors such as concentration, timing, and the specific microenvironment can influence whether a mediator acts as pro-inflammatory or anti-inflammatory. Some pro-inflammatory mediators play roles in resolving inflammation. For instance, certain types of prostaglandins, initially promoting inflammation, later contribute to the resolution phase. The immune system has feedback mechanisms where prolonged inflammation leads to the activation of anti-inflammatory pathways. Some cytokines, like IL-10, have dual roles in both promoting and inhibiting inflammation. Cytokines may switch roles by modulating signaling pathways. For example, TNF-α is primarily pro-inflammatory factor, but can induce anti-inflammatory effects under specific conditions. In some cases, mediators that cause inflammation in one tissue may have anti-inflammatory effects in another. The interaction of pro-inflammatory mediators with

other molecules in the immune system can modify their effects, leading to anti-inflammatory outcomes (Serhan and Savill (2005). Resolution of inflammation: the beginning programs the end. Nature Immunology, 6(12), 1191-1197; Lawrence and Gilroy (2007). Chronic inflammation: a failure of resolution? International Journal of Experimental Pathology, 88(2), 85-94; Aoki and Narumiya (2012). Prostaglandins and chronic inflammation. Trends in Pharmacological Sciences, 33(6), 304-311; Smith et al. (2000). Cyclooxygenases: structural, cellular, and molecular biology. Annual Review of Biochemistry, 69, 145-182; Nathan and Ding (2010). Nonresolving inflammation. Cell, 140(6), 871-882; Opal and DePalo (2000). Anti-inflammatory cytokines. Chest, 117(4), 1162-1172). These are all possible scenarios upon IR exposure of the brain and the lack of specificity and detail in the AOP 483 undermines its utility in hypothesis generation and knowledge gap identification.

Reply: We agree, while it is true that pro-inflammatory mediators, particularly certain cytokines, may exhibit dual roles with both anti-inflammatory and inflammatory effects, our focus on the pro-inflammatory responses is grounded in their impact on learning and memory impairment. The biological plausibility of this association is strong. Persistent oxidative stress leading to inflammation can lead to impairment of learning and memory. This is often associated with increased expression of pro-inflammatory markers, such as cytokines and chemokines, which can disrupt the long-term potentiation of synaptic plasticity required for learning and memory, leading to the activation of microglia and the release of more pro-inflammatory cytokines. The impaired resolution of inflammation in the brain can also lead to chronic inflammation and neuronal damage, further contributing to learning and memory deficits. In our data retrieval process, by considering pro-inflammatory mediators in the context of neural remodeling, studies have consistently shown that an increase in specific pro-inflammatory cytokines contributes to in neural remodeling and consequent cognitive deficits. However, we will highlight in the uncertainty section of the AOP report the dual role of inflammatory mediators.

The following has been added to the inconsistency section of the AOP report (page 28):

"Inflammatory markers can have both anti-inflammatory and pro-inflammatory roles. Factors such as concentration, timing, and the specific microenvironment can influence whether a mediator is pro-inflammatory or anti-inflammatory (Lawrence & Gilroy, 2007; Nathan & Ding, 2010; Opal & Depalo, 2000)."

The following has been added to the inconsistency section in the overall assessment section of the snapshot (page 8):

"Inflammatory markers exhibit a dual role, with the capacity for both anti-inflammatory and pro-inflammatory actions. Variables such as concentration, timing, and the specific microenvironment play pivotal roles in determining whether a mediator acts in a pro-inflammatory or anti-inflammatory manner (Lawrence & Gilroy, 2007; Nathan & Ding, 2010)"

Comment: Lack of discussion with respect to what brain regions are involved in each KE/KER; it is known that damage to different brain domains can impact learning and memory in distinct ways. The brain is a complex organ with various regions responsible for different aspects of learning and memory. Brain domains such as hippocampus, frontal lobes, temporal lobes,

parietal lobes, cerebellum and basal ganglia, amygdala all have distinct roles in learning and memory (The right parietal lobe is critical for visual working memory - PubMed (nih.gov); Human emotion and memory: interactions of the amygdala and hippocampal complex - PubMed (nih.gov)) This should at least be discussed, and ideally evidence from IR studies or studies covering the KER of the AOP should be presented.

Reply: Agreed that we can discuss this in more detail. Brain regions are discussed in the AOP report as follows (page 5):

"There are multiple brain areas involved in learning and memory including the hippocampal region, imperative for declarative or episodic memory and the process of long term potentiation, the amygdala, which can process emotional components to memory, the parietal lobe, which is involved in spatial memory, the prefrontal cortex, involved in regulating emotional behaviors, thoughts and actions and the basal ganglia, which may be important for stimulus response associations. These areas do not act independently as multiple brain areas may be involved at any given time depending on the task or stimulus (Berryhill & Olson, 2008; Cucinotta et al., 2014; Desai et al., 2022; NCRP Commentary, 2016; Phelps, 2004)."

Additionally, the learning and memory KE has information on brain regions relevant to the AO.

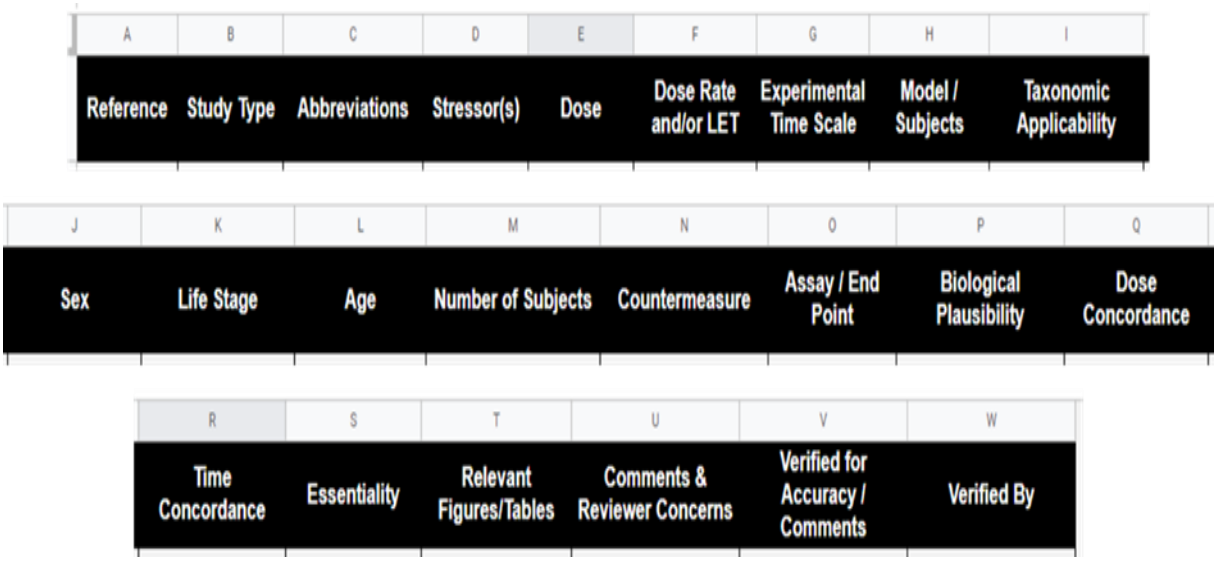
Comment: One aspect that the authors should consider including in the revised manuscript is the assessment of the relative amount of evidence that is supporting this AOP (positive evidence) vs. the evidence that is non-supporting (negative evidence). It is hoped that the literature screening and data extraction approach used by the authors would allow to carry out such assessment. This information appears to be very important for the identification of knowledge gaps and inconsistencies in a quantitative manner. Just as an example, a study by Chien et al (Low-dose ionizing radiation induces mitochondrial fusion and increases expression of mitochondrial complexes I and III in hippocampal neurons - PMC (nih.gov) could be mentioned where the finding suggest compensatory mechanisms at low, but not high dose of IR. Including such evidence in the assessment seems crucial: those KEs and KERs that would have the lowest ratio [positive/negative] or have low absolute number (not %) of positive evidence papers would be immediately tagged as knowledge gaps. Furthermore, this information, if related to the dose range, life stage and taxonomic applicability (shown in Fig 5) can provide unprecedented level of understanding of the relevance of biological mechanisms to human radioprotection scenarios (high vs. intermediate doses) and would inform future studies.

Reply: What the reviewer is suggesting is very interesting and could be the basis of another report that details the stressor related information across each KER, but it is beyond the scope of this paper. We do present an adequate overview of the composition of studies supporting the AOP. The PRISMA diagram (supplementary figure of the AOP report) provides details of excluded and included studies and the rationale for exclusion. Figures 3-6 provide an overview of the entire network and areas of knowledge gap including details of stressor information used to support the AOP. Furthermore, within each KER and the overall assessment presented in the snapshot, a weight of evidence call is provided based on biological plausibility, empirical support and quantitative understanding. Together this information highlights the knowledge gaps. Mechanisms related to low dose effects have been highlighted as a clear area of inconsistency in the overall assessment and within the AOP report.

In our review process, negative data, i.e., information that does not support the hypothesized associations or adverse outcome pathways, is carefully considered within the framework of the Bradford Hill criteria. Negative data, when analyzed against the Bradford Hill criteria, suggests the AOP lacks the strength, consistency, specificity, temporality, or biological plausibility required to establish causation. In such cases, we exercise caution in interpreting the findings, recognizing that the absence of evidence supporting a causal link is not necessarily evidence against it. However, to maintain the robustness and validity of our review, negative data that does not align with the Bradford Hill criteria may be excluded from the final synthesis. This exclusion ensures that the conclusions drawn are based on an evaluation of evidence that meets established criteria for causation, contributing to a more reliable and focused assessment of the relationships under consideration. Studies that are contradictory to the AOP are presented in the inconsistency and uncertainty section of the AO. The paper provided by the reviewer is a good example of inconsistency in results related to low dose effects, which has been added to the following KERs: MIE to oxidative stress, and MIE to neural remodeling.

Comment: Fig 5: How the data shown were calculated? And how they are distributed over the KERs? It would also be interesting to see somewhere in the AOP and the manuscript the number of included supporting studies that a) were done using non-IR stressors/treatments and b) were done in non-neuron/brain related models.

Excellent question. The reviewer is directed to the Kozbenko et al 2022 paper that is cited in the AOP report for details on the prioritization and selection criteria for inclusion and exclusion of studies. We use a three-tier process that involved inclusion and exclusion based on the Population, Exposure, Endpoint, and Outcome (PEOE) statement and then Bradford Hill (B-H) criteria. A PRISMA diagram is provided in the supplementary table. For each KER all studies supporting elements of the B-H criteria were tabulated in an Excel file (see below example). For each study passing the B-H criteria and PEOE statement, information on exposure parameters, domain of applicability and how the study supported the B-H criteria was tracked. This information was then used to generate the figures. Note that since some KERs are reused from other AOPs, that information was not included in the study number tabulation. Most studies used radiation and brain relevant cell types. This data is also summarized within the tables presented within each KER.



In light of the reviewer's comments, we have now added the following in the AOP report page 11-12:

"For all extracted studies information was tracked on exposure parameters, domain of applicability and how the study met the Bradford Hill criteria. This extracted data was used to generate visual graphics that provide a summary of dose range, stressor types, domain of applicability and evidence stream used to support the AOP."

Comment: Fig. 6: There are several questions here: a) why some parts do not have low dose label; how to find them, they are not in order (low-intermediate-high) for each section? B) what are unlabeled zones (question marks on the screen shot below)?

Reply: Thank you for noting this, the figure has been revised.

Comment: Suggestions for Table I: Consider adding the following methods for KE 1392: a) Chemiluminescence: This method involves luminescent probes that emit light when they react with ROS. The light intensity is proportional to the ROS level, providing a direct measure of oxidative stress. B) Electron Spin Resonance (ESR) Spectroscopy: This technique directly detects free radicals by measuring their unpaired electrons using magnetic fields and radiofrequency. It's considered the gold standard for direct free radical measurement.

Reply: Agreed. Both methods of measurements have been added to Table I under KE 1392.

Comment: Page 24, lines 3-9: It seems that this uncertainty applies to many other parts of the AOP and may not be listed under bullet #2

Reply: Agreed. The following has been updated in the AOP report on page 26:

"Empirical evidence supporting tissue resident cell activation following an increase of oxidative stress is inferred exclusively from gamma radiation studies. A knowledge gap exists regarding the impact of other forms of exposure."

Comment: Page 24, lines 23-34: This uncertainty/inconsistency should be extended to KEs 2066, 1492 and 1493. They also have a wide range of readouts and markers that can be used to define them.

Reply: Agreed. We now add the following in the uncertainty section page 28 of the AOP report:

"The utilization of diverse assays to assess KEs may result in variations in the quantitative interpretation of observations across studies."

Comment: Page 29: sentence "the AOP could be part of the literature evaluation used to consider the reclassification of health effects from radiation exposures" should be clarified

Reply: We have clarified. The following has been added to the AOP report page 32:

"The AOP presented could serve as an integral component in the consideration of reclassifying health outcomes attributed to radiation exposure. Through evidence-based understanding of the associated risks and outcomes, the AOP structured framework could aid regulatory bodies and international governing bodies in re-evaluating and potentially refining the classification of health effects related to radiation exposures."

REVIEWER COMMENTS (ROUND 2)

This reviewer is grateful to the authors for addressing the comments provided. While the revised manuscript is overall improved, some comments were addressed incompletely. The major remaining overarching concern is the lack of specificity for some Key Events (KEs), including their description and methods of detection. Details are as follows:

Key Event 3: 2066, Altered Signaling Pathways: The authors write, "However, in light of the reviewer's comments we have revised the KE description to be more specific regarding some pathways that when dysregulated are associated with disease processes. Please refer to page 45 of snapshot." Careful examination of the revised description on page 45 did not change the opinion of this reviewer that it does not provide specific enough information on the signaling pathways relevant to the AOP. Reference to cancer is irrelevant, as is most of the revised text. The authors cite "best practices in AOP development" in support of their broad choices; however, the same "best practices" can be applied in support of the comment, e.g., the requirement of measurability and scientific rigor. As an illustration of this point, let's consider measurability in the following scenario: a 2-fold increase in p53 protein levels is detected in a brain biopsy (the authors have marked activation/upregulation of p53 as an endpoint for the KE). Does that mean that the KE 'Altered signaling pathways' has occurred? The response of this reviewer would be 'No.' It is common knowledge that stabilization of the p53 protein and its transcriptional activation can indeed occur in response to stress. In fact, it is a key regulator of orchestrating the attempt to repair DNA damage and, if that fails, trigger apoptosis, and if that fails, activate further signaling that MAY lead to senescence (as one of the pathways in KE 2066). This essentially means that activation of p53 is more relevant to DNA damage sensing/signaling and repair and has weak relevance to senescence signaling leading to neural remodeling. What is the critical level of measurable endpoints and their number that would suffice for qualifying the KE as occurring? It seems that no such level can be determined/proposed because of the vagueness of the KE and its description, thus undermining the scientific rigor and, most importantly, the perspectives for converting the AOP to a quantifiable one. This is just one of many possible illustrations of how the KE 'altered signaling pathways' undermines the many advantages and added values of the AOP concept. It is thus still the opinion of this reviewer that the KE should be narrowed down to be more specific to the AO in question.

Furthermore, examination of the endorsed AOPs on AOP-Wiki that use the same AO "Impaired learning and memory" reveals that those AOPs contain elements that are much more specific to neural/brain effects and biology. When the elements are applicable to other tissues and organs, it is stated. An explicit example is AOP-17, which shares the AO and KE 1492 "tissue resident cell activation" with the AOP of this manuscript. The difference between the descriptions of KE 1492 "tissue resident cell activation" and KE 2066 "Altered Signaling Pathways" is quite clear: the former is specific, and the latter is generic. The same is evident for other AOPs with the AO "Impaired learning and memory." It is thus in the best interest of the authors to revise the KEs (2066 and 2098) that are very broadly defined to facilitate subsequent endorsement.

Another relevant but distinct comment concerns the methods of detection. Specifically, for KE 2066, the table with methods should be revised. It is non-informative (and probably not acceptable) to list generic techniques in this section. Each of those listed can be used to measure a myriad of processes, but what exactly presents the measurement of a KE or a biological process in a KE must be stated. Unless specific antibodies, dyes, and other details are provided, this information is useless. This is probably a consequence of the broad nature of the KE itself.

One specific comment regarding the methods is that page 64 of the 'snapshot' refers to an MRI method as one that detects demyelination. In fact, MRI in the cited paper is not used to detect demyelination; it is used to detect necrotic volume in the brains of 45Gy irradiated mice. It is hardly applicable to the irradiation dose and context (a 45Gy irradiated human will not be subjected to a test to assess the risk of memory and cognition impairment); the relevance of necrosis to the utility of the AOP is also very questionable. It is impractical for reviewers to examine each reference used for validity, so the authors are advised to verify the references in this regard.

Lastly, the authors write, "Most KEs (new KEs include #2066 & 2098) in our AOP are reused from existing endorsed AOPs in the AOP Wiki. This also includes the AO. Thus, this limits the extent of changes that can be made to them. AOPs are built in a modular fashion to ensure that KEs and KERs are shared between AOPs. This is a core principle that we are required to adhere to in AOP development so that networks can emerge in the future." This is all correct. However, another core principle of AOP development is that they have to be based on scientific rigor. Thus, the existence of endorsed KEs and AOs does not by itself constitute justification for their use or their use as is. The authors may also be aware that the current reassessment of the AOP platform includes consolidation and normalization of terminology and other components to improve the ability to construct AOP networks. Therefore, it should be feasible and acceptable to consider a new title and thus description for a KE that already exists but undermines the scientific rigor and utility of the AOP in question.

AUTHORS RESPONSE TO REVIEWER COMMENTS (ROUND 2)

A conference call was arranged between the authors and reviewer to address the outstanding round 2 reviewer comments on July 9, 2024. The author and reviewer agreed to the changes in KE #2066 proposed by the author below:

KE #2066 –Altered, Stress Response Signaling – KE Description

Biological context

Level of Biological Organization
Molecular

Key event components

Process	Object	Action
Cellular signaling		Altered

Key event overview

Prototypic Stressors

Name
Ionizing radiation
Altered gravity

Taxonomic Applicability

Term	Scientific term	Evidence	Link

Human	Homo sapiens	Moderate	NCBI
Rat	Rattus norvegicus	Moderate	NCBI
Mouse	Mus musculus	Moderate	NCBI

Life stages

Term	Evidence
All stages	Moderate

Sex applicability

Term	Evidence
Unspecific	Low

Key event description

Cells rely on a balance of signaling pathways to maintain their functionality and viability. These pathways integrate signals from both external and internal stressors to coordinate protective responses, thereby enhancing the cell's ability to cope with adverse conditions. Key components of these pathways include the activation of stress-responsive transcription factors such as NF-κB, p53, and AP-1, which regulate the expression of genes involved in cell cycle arrest, DNA repair, and apoptosis. DNA double-strand breaks, for instance, initiate a cascade of events involving the ataxia-telangiectasia mutated (ATM) kinase, the DNA-dependent protein kinase (DNA-PK), and the p53 pathway, ultimately leading to cell cycle arrest and repair mechanisms or apoptosis if the damage is irreparable (Kastan and Lim, 2000). Furthermore, the mitogen-activated protein kinase (MAPK) pathways, including ERK, JNK, and p38, are crucial for the cellular stress response and inflammatory processes (Dent et al., 2003).

These pathways are essential in regulating cellular survival and mediating apoptosis under various physiological and pathological conditions. Persistent signaling or a pre-existing inflammatory

environment can significantly influence cell fate. For instance, the cAMP-PKA pathway, which is involved in neurotransmitter signaling, impacts synaptic plasticity and memory formation (Zhang et al., 2024). The MAPK pathway, encompassing ERK, JNK, and p38 MAP kinases, is vital for cell differentiation, proliferation, and response to stress stimuli (Arthur and Ley, 2013; Yue and Lopez, 2020). The PI3K-Akt pathway promotes cell survival and growth by inhibiting apoptotic processes and supporting metabolic functions (Manning and Cantley, 2007). The p53 pathway is a key regulator of the cellular stress response, often leading to apoptosis in the context of severe DNA damage or oxidative stress (Kruiswijk et al., 2015).

Exposure to stressors, such as radiation, can disrupt these signaling pathways or lead to persistent activation. For example, the cAMP-PKA pathway can be hindered by reduced cAMP levels and impaired PKA activity, leading to decreased CREB phosphorylation (Zhang et al., 2024). The MAPK pathway is affected by external stressors through the inhibition of ERK activation and subsequent gene expression (Kim and Choi, 2010). The PI3K-Akt pathway, which is vital for cell survival, experiences reduced PI3K activity and Akt signaling, impairing mTOR-mediated protein synthesis (Glaviano et al., 2023; Martini et al., 2014). Activation of the p53 pathway in response to DNA damage can also potentially induce cellular senescence if the damage is irreparable (Ou et al., 2018). Persistent disruptions in these pathways can lead to a wide range of pathophysiological conditions, including neurodegenerative diseases, chronic inflammation, cardiovascular disease, and cancer.

Key Stress Response Pathways: Description and Components for Measurement

AMP-PKA Pathway:

The AMP-PKA pathway is activated by stressors which engage G protein-coupled receptors (GPCRs), GPCRs activation leads to the production of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase. cAMP then goes on to activate protein kinase A (PKA), which is one of the primary kinases required for several functions in the cell such as DNA repair and initiating a response to oxidative stress. (Hunter, 2000; Jessulat et al., 2021; Steinberg and Hardie, 2023). This results in PKA phosphorylating various target proteins, thereby influencing gene expression, metabolism and cell survival.

MAPK Pathway:

MAPK pathway is triggered by a variety of stressors, including growth factors, cytokines, hormones and various cellular stressors such as oxidative stress (Kim and Choi., 2010). The pathway involves a kinase cascade starting from receptor tyrosine kinases (RTKs) or GPCRs, leading to the activation of Ras, Raf, MEK, and ERK. Activated ERK then translocates to the nucleus and regulates gene expression, affecting cell growth, differentiation, and apoptosis (Morrison, 2012).

PI3K-Akt Pathway:

The PI3K-Akt pathway is activated by stressors through receptor tyrosine kinases (RTKs) or GPCRs. Activation of phosphoinositide 3-kinase (PI3K) generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), recruiting and activating Akt. Akt then phosphorylates downstream targets, resulting in promotion of cell survival, growth, and metabolism while inhibiting apoptosis (Martini et al., 2014; Jin et al., 2022).

NF-κB Pathway:

NF- κB is activated by pro-inflammatory cytokines, pathogens, and stress signals. This pathway involves the activation of IκB kinase (IKK), which phosphorylates IκB, leading to its degradation and the release of NF-κB. NF-κB then translocates to the nucleus and promotes the expression of genes involved in inflammation, immune response, and cell survival (Liu et al., 2017)

JAK-STAT Pathway:

The JAK-STAT signalling pathway is triggered by cytokines and growth factors. Janus kinases (JAKs) are then activated, which phosphorylate and activate signal transducer and activator of transcription (STAT) proteins. Activated STATs dimerize and translocate to the nucleus to regulate gene expression, impacting cell proliferation, differentiation, and immune function. This signalling pathway is involved in multiple important biological processes such as differentiation, apoptosis, cell proliferation and immune regulation (Xin et al., 2020).

HSP (Heat Shock Protein) Pathway:

HSP (Heat Shock Protein) pathway is induced by heat shock, oxidative stress, and other proteotoxic stresses. Stress signals lead to the activation of heat shock factor 1 (HSF1), which

translocates to the nucleus and promotes the expression of heat shock proteins (HSPs). HSPs act as molecular chaperones, aiding in protein folding, preventing aggregation, and promoting protein degradation. These proteins can also work as danger signalling biomarkers, being secreted to the exterior of the cell in response to stress (Zininga et al., 2018)

p53 Pathway:

The p53 pathway is activated by DNA damage, oxidative stress, and other genotoxic stresses. DNA damage activates kinases like ATM and ATR, which phosphorylate and stabilize p53. p53 then regulates the expression of genes involved in cell cycle arrest, DNA repair, and apoptosis (Joerger and Fersht, 2016). p53 functions also expand to roles in development, metabolic regulation and stem cell biology.

Unfolded Protein Response (UPR):

Unfolded Protein Response (UPR) is triggered by the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) (Hetz et al., 2020). This pathway involves sensors such as IRE1, PERK, and ATF6, which detect ER stress and activate downstream signaling pathways (Ron and Walter, 2007). UPR aims to restore ER homeostasis by enhancing protein folding capacity, degrading misfolded proteins, and reducing protein synthesis (Grootjans et al., 2016).

Method of detection/measurement:

Pathway	Method of Measurement	Description	Reference	OECD Approved Assay
cAMP-PKA	ELISA	Measures intracellular cAMP concentrations to assess activation of the cAMP-PKA pathway.	Zhu et al., 2016	No
	cAMP-Glo™ Assay	Monitors the level of intracellular cAMP in the cell with receptors that are modulated by lipid	Hu et al., 2019	No

Pathway	Method of Measurement	Description	Reference	OECD Approved Assay
		and free fatty acid agonists.		
	Western Blot	Detects phosphorylation of PKA substrates, indicating pathway activation.	Zhang et al., 2021	No
	Direct cAMP Enzyme Immunoassay	Uses a cAMP polyclonal antibody to competitively bind the cAMP in the sample which has cAMP covalently bonded.	Nogueira et al., 2015	No
	RT-PCR	Quantifies mRNA levels of PKA-RII and PKA-C.	Zhu et al., 2016	No
MAPK	Western Blot	Detects the phosphorylation state of MAPK family members (ERK, JNK, p38), indicating activation.	Tan et al., 2022; Xia and Tang 2023	No
	Immunohistochemistry	Visualizes the activation of MAPKs (JNK and p38) in tissue sections using specific antibodies.	Er et al., 2022	No
	qRT-PCR	Quantifies mRNA levels of JNK, MAPK1(ERK), and MAPK14(p38)	Xia and Tang 2023	No
PI3K-Akt	Western Blot	Detects phosphorylation of proteins such as PI3K and AKT.	Jin et al., 2022; Xia and Tang 2023; Bamodu et al., 2020	No

Pathway	Method of Measurement	Description	Reference	OECD Approved Assay
	qRT-PCR	Quantifies mRNA levels of AKT1 and PI3K.	Xia and Tang 2023	No
p53	Western Blot	Measures levels of p53 and its downstream target proteins to assess activation.	Wei et al., 2024, Mendes et al. 2015	No
	qPCR	Quantifies mRNA levels of p53-regulated genes such as p21, Bax, and H3K27me3.	Wei et al., 2024	No
	Chromatin immunoprecipitation (ChIP)	Detects p53 binding to DNA at target gene promoters.	Vousden and Prives, 2009; Wei et al., 2024	No
	Co-immunoprecipitation (Co-IP)	Identifies p53 protein to protein interactions.	Wei et al., 2024	No
	Immunofluorescence	Visualizes localization and expression of p53.	Wei et al., 2024	No
NF-κB	Western Blot	Detects phosphorylation and degradation of IκBα, indicating activation of the NF-κB pathway.	Mao et al., 2023; Meier-Soelch et al., 2021; Xia and Tang 2023	No
	Electrophoretic Mobility Shift Assay (EMSA)	Measures DNA-binding activity of NF-κB to specific response elements.	Meier-Soelch et al., 2021; Ramaswami and Hayden, 2015	No
	ELISA	Quantifies NF-κB DNA-binding activity in nuclear extracts.	Meier-Soelch et al., 2021	No

Pathway	Method of Measurement	Description	Reference	OECD Approved Assay
JAK-STAT	Western Blot	Measures levels of JAK2 and STAT3	Broughton and Burfoot, 2001; Mao et al., 2023	No
	Electrophoretic Mobility Shift Assay (EMSA)	Measures DNA-binding activity of STAT proteins to specific response elements.	Broughton and Burfoot; Jiao et al., 2003	No
HSP	Western Blot	Measures levels of heat shock proteins such as HSP70 and HSP83.	Kaur and Kaur, 2013; Thakur et al., 2019	No
	ELISA	Quantifies levels of specific heat shock proteins in cell extracts.	Kaur and Kaur, 2013	No
	Immunofluorescence	Visualizes localization and expression of heat shock proteins in cells.	Thakur et al., 2019	No
UPR	Western Blot	Measures levels of UPR markers such as PERK, IRE1α, ATF-6	Sita et al., 2023, Kennedy et al., 2015; Zheng et al., 2019	No
	qPCR and RT-PCR	Quantifies mRNA levels of UPR-regulated genes such as ATF4 and CHOP.	Kennedy et al., 2015; Zheng et al., 2019	No
	Immunofluorescence	Visualizes localization and expression of UPR markers in cells.	Zheng et al., 2019	No

Domain of Applicability

Taxonomic applicability: Altered signaling is applicable to all animals as cell signaling occurs in animal cells. This includes vertebrates such as humans, mice and rats (Nair et al., 2019).

Life stage applicability: Life stage applicability is pathway dependent.

Sex applicability: This key event is not sex specific.

Evidence for perturbation by a stressor: Multiple studies show that signaling pathways can be disrupted by many types of stressors including ionizing radiation and altered gravity (Cheng et al., 2020; Coleman et al., 2021; Su et al., 2020; Yentrapalli et al., 2013).

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