**Adverse Outcome Pathway (AOP) External Review Report**

**Title: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment (AOP 48)**

**OECD Project 1.23**

***AOP Reviewers:***

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(Regulatory)

**Cristina Suñol, PhD**

Neurotoxicologist and Chief of the Neurotoxicity Group

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(Government/Basic Neurotoxicity)

**Kathleen Raffaele, PhD**

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Neurotoxicologist

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**Timothy J. Shafer, PhD**

Neurotoxicologist

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**Didima M.G. de Groot, PhD, ERT**

Senior Scientist/Consultant Developmental Biology/Neurotoxicology and Safety Pharmacology

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(Academic and Regulatory)

**AOP Authors:**

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

**Reviewers Comments (here onwards “comment”):**

*1a. Mitochondrial dysfunction can also be detected by mitochondrial fragmentation, using immunohistochemical techniques. One reviewer suggested citation of the following two references.*

Knott AB, Perkins G, Schwarzenbacher R, Bossy-Wetzel E. (2008) Mitochondrial fragmentation in neurodegeneration. Nature reviews Neuroscience. 9(7):505-518.

Sekino Y, Tanaka S, Hanamura K, Yamazaki H, Sasagawa Y, Xue Y, Hayashi K, Shirao T. (2006) Activation of *N*-methyl-D-aspartate receptor induces a shift of drebrin distribution: Disappearance from dendritic spines and neuronal network function in adult brain, and appearance in dendritic shafts. Mol Cell Neurosci. 31(3):493-504.

*1b. Overactivation of NMDARs can be detected by immunohistochemical technique for drebrin (an actin binding protein located in dendritic spines of matured hippocampal and cortical glutamatergic neurons) more easily than the whole-cell clamp recording.*

**Authors Response (here onwards “response”):**

Additional assays for evaluation of the mitochondrial dysfunction and overactivation of NMDARs have been addressed and the suggested references have been cited.

**Comment:**

1. *Intracellular Ca2+ overload leads long-term depression (LTD) in the excitatory synapses and it should be included in the AOP.*

**Response:**

LTD has been described, including LTD-induced synaptic dysfunction (triggered by NMDAR overactivation) in key event (KE) *NMDARs Overactivation* and in key event relationship (KER) *Impaired Neuronal Network Function*, supported by the relevant literature. The authors decided to keep long-term potentiation (LTP) as well, because of its relevance to NMDARs.

**Comment:**

*2a. Domoic acid (DomA) indirectly activates the NMDARs (not directly), by releasing glutamate. Since DomA is not an agonist of the NMDAR, it is suggested that the subtitle be changed to “Overactivation of the NMDARs.”*

*2b. The molecular initiating event* (*MIE) for this pathway is not binding of DomA to the NMDA receptor, but binding to the kainic acid (KA)/α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptor. One reviewer suggests redefining the initial steps of this AOP, by either lessening the focus on the NMDA receptor, or document compounds that are direct agonists of the NMDARs.*

**Response:**

As described in this AOP, DomA indirectly activates the NMDARs by releasing glutamate after binding to KA/AMPA receptors. The title of this AOP has been changed to: *Binding of agonists to inotropic glutamatergic receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.* Accordingly, the text describing the role of KA and AMPA receptors in NMDARs activation in the MIE and in the process of excitotoxicity has been changed. Also, the text of an abstract and, where relevant, the description of KEs is updated. Additionally, wherever possible, the available results after exposure to another chemical, Glufosinate (an herbicide) have been inserted. However, not all KERs are covered due to lack of data.

**Comment:**

*3a. In ‘Neural networks’ section, transmitter systems, other than glutamatergic or NMDARs, are not discussed. Further, ‘How it is measured or detected’ section needs to be more focused on individual circuits than on networks.*

 *3b.**Examples of the use of neurodegeneration as a regulatory endpoint is lacking.*

**Response:**

As suggested, more text is inserted to describe the role of additional transmitters in the context of neuronal network function.

The definition of dementia is expanded and the factual statements related to treatment or prevention of neurodevelopmental disorders are substantiated with relevant references. Also, the regulatory context is updated.

**Comment:**

1. *In the list of regulatory examples, only European Union (EU) regulations are cited, and the United States (US) regulations need to be included.*

**Response:**

The US relevant regulations have been included.

**Comment:**

1. *Microelectrode arrays (MEAs) can be used to examine glutamatergic neurotransmission, they do not specifically measure NMDAR activity. This needs to be clearly stated in the AOP.*

**Response:**

The application ofMEAs for not only the measurements of spontaneous activity, but also evoked activity, by applying specific agonists and antagonists of NMDA receptors has been discussed. The suggested papers (Frega et al., 2012 and Lantz et al., 2014) are cited.

**Comment:**

1. *In the context of species differences in susceptibility to DomA, sensitivity of humans deserves special attention.*

**Response:**

The sensitivity of humans to DomA induced toxicity has been expanded.

**Comment:**

1. *The reviewers have made a suggestion of including several relevant references in this AOP, in addition to technical and typographical errors.*

**Response:**

The relevant references have been included in the revised AOP. Also, the revised AOP has been reviewed and corrected by an English proficient reader.

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

**Comment:**

1. *One reviewer suggests changing key events.*

*Order of key events should be NMDARs, binding of agonist -> NMDARs, overactivation -> Ca influx, increased -> Mitochondrial dysfunction -> Cell injury/death -> Neurodegeneration.*

*Overactivation of NMDARs directly leads to synaptic dysfunction before cell death. The overactivation of NMDARs is known to induce LTD in the hippocampal slices. LTD is a model of synaptic dysfunction. Thus, neuronal network function can be impaired after overactivation of NMDARs. Not only after cell death and neurodegeneration, decreased neuronal network function can be led by intracellular Ca2+ overload through NMDARs.*

**Response:**

LTD-induced synaptic dysfunction (triggered by NMDAR overactivation) is now discussed in KE *NMDARs Overactivation* and in KER *Impaired Neuronal Network Function*, supported by the relevant literature.

**Comment:**

1. *One reviewer raises a concern of estimating a threshold of each key event which can determine if the key event will lead to the next key event or not.*

**Response:**

Authors state that it is difficult to estimate a threshold for each KE, as such data are not available. However, the authors have introduced a threshold for Ca2+ overload.

**Comment:**

1. *One reviewer suggests that overactivation of NMDAR and Ca2+ influx / Ca2+ overload must be on the top of the key events list. And consequently, the sections of NMDAR overactivation and Ca2+ overload should be placed ahead of mitochondrial dysfunction.*

**Response:**

The incorrect order of KEs and KERs in this AOP are due to IT issue, and hopefully it will be corrected as soon as possible. Since the title of this AOP has been changed, the description of MIE and the relevant KEs has changed accordingly.

**Comment:**

 *4a. One reviewer has some concern that the AOP is based almost entirely on data for a single chemical DomA, which activates receptors other than NMDARs. Also, how do we know that only NMDARs are activated?*

 *4b. The reviewer agrees with overview scheme of the AOP and the weight of evidence for the different KEs and KERs supported by convincing data, but much of the data is based on DomA only.*

**Response:**

TheAOP is mainly based on DomA since there is significant amount of information on the mechanism of toxicity, including human exposure. In the revised AOP, the authors have described the role of AMPA and KA receptor activation in triggering NMDA receptor overactivation. Sustained NMDARs activation leads to the overloading of cells with Ca2+ that is one of the critical KEs in DomA induced toxicity.

To make stronger, the authors have included in this AOP existing data on Glufosinate exposure through all KERs, as an example of the chemical that acts through direct activation of NMDARs, causing convulsions and amnesia (AO). Furthermore, the role of neuroinflammation as suggested is now evaluated as ‘weak’ in KEs table.

**Comment:**

1. *A more detailed discussion of the quantitative relationships between NMDAR activation and Ca2+ influx and Ca2+ overload is needed to strengthen the KER. A similar discussion is also needed on the quantitative relationships between Ca2+ homeostasis, mitochondrial dysfunction, and cell death.*

**Response:**

The authors agreed with reviewer’s assertion that the definition of a threshold for each KEup that triggers KEdown is critical for quantitative AOP evaluation. However, at this stage due to lack of data, this AOP remains mainly qualitative. The authors have added some information about a threshold of intracellular Ca2+ levels that trigger transition from physiological to pathological state of the cell (discussed in KER).

**Charge Question 3: *What would be the regulatory applicability of this AOP in your opinion?***

**Comment:**

*1a. The applicability of this AOP is for those compounds that can cross the blood-brain barrier and interact with NMDARs and KA receptors. This AOP explains the deficits in learning and memory found in DomA intoxicated people.*

*1b. One reviewer suggests additional in vitro testing or regulation in terms of quantitative information regarding the level of binding affinity or receptor occupancy of NMDAR agonists.*

*1c. One reviewer recognizes the potential of this AOP in the context of overt behavioral and memory alterations associated with DomA poisoning in humans. However, its regulatory aspect can be further strengthened by inclusion of environmentally relevant chemicals or anthropomorphic compounds.*

**Response:**

This AOP is mainly based on the available qualitative information, but that is due to lack of quantitative information. The authors hope that *in vitro* testing strategy can be built based on the KEs identified in this AOP. The critical assay would be for MIE. There are available methods for quantitative evaluation of an agonist binding affinity and NMDAR occupancy. Such assay could be used for the screening purposes to identify those chemicals that have potency to behave as KA/AMPA and/or NMDA receptor agonists. Relevance of this AOP is further strengthened by including Glufosinate, which triggers neurotoxicity through direct activation of NMDARs. Unfortunately, the empirical support describing the effects of exposure to this herbicide is not available to support all KERs.

**Charge Question 4: Overall assessment of the AOP-*Would you recommend this AOP to be submitted to WNT and TFHA for endorsement?***

**Comment:**

1. *The reviewers suggest recommendation of this AOP to WNT and TFHA pending a suitable revision.*

**Response:**

The authors have addressed all reviewers concerns and a satisfactory revised AOP is being revised and/or finalized.

**Conclusion**:

 As review manager, I would like to commend the authors of this AOP, which deals with a very timely topic. Also, I would like to appreciate the reviewers for their constructive comments and suggestions. This report is prepared based upon information that I gathered from the reviewers, authors, teleconferences and AOPWIKI. All reviewers are satisfied with authors’ responses and all key issues are resolved.

Respectfully submitted to OECD Secretariat by:

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