EXTERNAL REVIEW REPORT

AOP-12

Title: Chronic binding of antagonist to *N*-methyl-*D*-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging

Introduction and Background

The AOP 12 is based on two previous AOPs (AOP 13 and AOP 48) dealing with NMDAR agonists and antagonists. Chronic NMDARs inhibition during brain development by lead (Pb^{2+}), a developmental neurotoxicant, leads to adverse outcomes, including neurodegeneration in hippocampus and cortex with amyloid plaque deposition and tau hyperphosphorylation and impairment of learning and memory, which are considered hallmarks of Alzheimer's disease (AD). Although this AOP includes multiple KEs, neurodegeneration and neuroinflammation remain the focal points. Whether neurodegeneration precedes neuroinflammation or neuroinflammation precedes neurodegeneration still remains a central issue to be resolved due to lack of sufficient evidence. Long temporal delay between exposure and adverse outcome is a real difficulty and challenge for neurotoxicity testing; and the proposed AOP suggests multiple models for neurotoxicity testing that can be utilized for regulatory purposes.

External reviewers: April Kluever, Hester Hendriks, Kaoru Sato, and Henri Schroeder

Authors: Florianne Tschudi-Monet, and Rex FitzGerald

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

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

Comment-1: All reviewers complimented the authors/developers for incorporating sufficient scientific knowledge from peer-reviewed literature in this AOP, as it clearly describes the pathway from the molecular initiating event up to organ and organism effect. However, in general, more neurotoxicological *in vitro* assays need to be highlighted as these techniques will be used in the near future for regulatory purposes instead of the traditional *in vivo* neurotoxicity tests. The reviewers suggested inclusion of electrophysiological and behavioral tests.

Response: *Authors have added the suggested electrophysiological and behavioral tests in the KE "impairment of learning and memory", in the subchapter "How is it measured or detected".*

Comment-2: The reviewers requested inclusion of the discussions, suggestions and recommendations as mentioned in the recent paper by Aschner *et al* (2017) Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential

of chemicals: Example lists and criteria for their selection and use, Altex 2017. https://doi.org/10.14573/altex.1604201.

Response: *The authors included the reference by Aschner et al (2017) and the responses provided by the authors were adequate and no further actions are deemed necessary.*

Comment-3: The reviewers requested the AOP authors to consider providing evidence that lead (Pb^{2+}) exposure results in neurological decline, or whether it increases the uncertainty regarding developmental exposure being the critical window for subsequent effects on neurological decline in later life. The paper by Schwartz BS, *et al* (Past adult lead exposure is associated with longitudinal decline in cognitive function, *Neurology* **55** (2000), 1144-1150) was suggested for inclusion.

Response: *The authors included the reference by Schwartz et al (2000) and adequately responded to the reviewers query and no further actions are deemed necessary.*

Comment-4: The reviewers suggested moving the last sentence of the Abstract to the Optional Background-Section. Also, this seems to be the right place for citing the Aschner *et al* (2017) paper, which provides an overview of useful test methods.

Response: *The last sentence of the abstract was moved to the background part of the Overall Assessment of the AOP, with the reference of Aschner et al (2017). The reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

Comment-5: The reviewers noted in section 3.3 KEs, 52: Decreased, calcium influx; How it is measured or detected (Page 9) that several methods for measurement of intracellular calcium homeostasis are mentioned. In the ongoing discussion on the assessment of real-time kinetic changes in intracellular calcium, either a plate-reader-based-approach or fluorescence microscopy needs to be included. Off course, the plate-reader-approach has been criticized because of its lower resolution, sensitivity, and other factors (Meijer M., Hendriks HS, Heusinkveld HJ, *et al*. 2014. Comparison of plate reader-based methods with fluorescence microscopy for measurements of intracellular calcium levels for the assessment of *in vitro* neurotoxicity, *Neurotoxicology 45: 31-37*; http://dx.doi.org/10.1016/j.neuro.2014.09.001). In the same section, the reviewers also noted that the indicators for calcium homeostasis are lacking (Vijverberg HPM and Westerink RHS. 2012. Sense in Pb^{2+} sensing, *Toxicol Sci*. 130(1): 1-3; https://doi.org/10.1093/toxsci/kfs221).

Responses: *The two suggested methods have been added in KE; decreased calcium influx, as suggested by the reviewers. During the TC, the reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

Comment-6: In section 3.3, KEs 55: N/A, Cell injury/death; How it is measured or detected (Page 14), the reviewers suggested that the AOP authors also consider implementing other cell viability tests, such as a combined Alamar Blue (mitochondrial activity) and Neutral Red (membrane integrity and lysosomal activity) assay. The ATP assay is also popular to evaluate cell viability (CellTiter-Glo[Promega], etc). AO/EB staining is also used to detect apoptosis (Cold Spring Harbor Protocols 2006(3), August 2006). Further, in the same section, KEs 188: N/A, Neuroinflammation; How it is measured or detected (Page 16), no methods for measurement of ROS/oxidative stress are mentioned. The reviewers suggested that oxidative stress can be measured in neuronal cells (*in vitro*) over time using the H_2-DCFDA assay (LeBel CP and Bondy SC. 1990. Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. *Neurochem Int*, 17(3): 435-440). The reviewers also suggested supplementing the information on quiescent microglia expressing CD11, Iba1, etc. Inflammatory microglia can be determined by the increased expression levels of these markers together with the morphological changes.

Response: *KE 52: Cell injury/death. How is it measured? As suggested by the reviewers, four proposed assays have been added. During the TC, the reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary. KE 188: Neuroinflammation. No further action is needed. The fact that reactive microglial cells can be detected by increased expression levels of markers such as CD11, Iba1, and IB4 was already present in the KE description. The morphological changes have been added, as suggested by the reviewers. The reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

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

In general, the reviewers concur with the judgement and scoring provided by the AOP developers. However, the reviewers have raised many issues, which are as follows:

Comment-1: The reviewers requested more information on the rationale of classification of the weight of evidence, especially when it is stated as moderate or weak. It is not clear why KER Cell injury/death leads to N/A, Neuroinflammation (Page 35) is classified as “weak”.

Response: *During the TC, authors clarified and reiterated that KER Cell injury leads to Neuroinflammation was classified "moderate", not "weak". This classification has been based on the facts that: (1) there is not enough specific literature showing that blocking Pb^{2+} -induced cell death/injury in immature brain abolished the inflammatory response, and (2) cell death is not the only trigger of neuroinflammation, several other types of injury (like decrease of specific neuronal features due to neurotoxic stress or to blockage of neuronal differentiation) can also trigger glial reactivity. The reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

Comment-2: The reviewers pointed out that the "Binding antagonists to NMDARs" sounds unclear. Does "antagonists" involve competitive, non-competitive antagonists and channel blockers as well? Taking the case of Pb^{2+} into consideration, Pb^{2+} exerts its effects through mechanisms other than antagonistic effects on NMDARs. Furthermore, the reviewers wanted to know which of the two mechanisms is more important.

***Response**: This KE developed for AOP 13 has already been through the external review process. The issue has been discussed with Anna Price, the corresponding author of AOP 13. Pb^{2+} binds to NMDAR as a non-competitive antagonist. The authors agreed with the fact that Pb^{2+} can exert its adverse effects through mechanism(s) other than antagonistic effects on NMDARs, but it is the principle of AOP to consider one possible pathway and to add the other possible mechanisms in the uncertainties. The authors have described the other mechanisms of toxicity of Pb^{2+}, and accordingly, they will add these in the KER Binding of antagonist of NMDAR leads to inhibition of NMDARs. In the context of additional mechanisms of toxicity, Pb^{2+} has been shown to cause oxidative stress. Furthermore, Pb^{2+} has the ability to substitute other bivalent cations like Ca^{2+},Mg^{2+}, and Fe^{2+} and monovalent cations like Na^{+} (for review, see Flora et al., 2012). Flora G, Gupta D, Tiwari A. (2012) Toxicity of lead: A review with recent updates. Interdisciplinary Toxicology 5(2): 47-58.*

Comment-3: The reviewers requested clarification for the binding sites for Pb^{2+} in NMDAR (Page 3, lines 18-19).

***Response:** The following sentence and reference have been added in KER under empirical support: Pb^{2+} acts as a non-competitive, voltage- independent antagonist of NMDAR. Pb^{2+} action partially overlaps with that of Zn^{2+} (Gavazzo et al., 2007).*

*In addition, this was already present in AOP wiki: **Pb^{2+}:** Although the NR2 subunits have different Zn^{2+} binding sites, i.e. the NR2A-NMDAR binds Zn^{2+} at a high-affinity site (nM affinity), while the NR2B-NMDAR binds Zn^{2+} with lower affinity (µM range); the Pb^{2+} IC_{50} for wild type NR2A-NMDARs was reported to be 1.3 µM, while the Pb^{2+} IC_{50} of wild type NR2B-NMDARs was 1.2 µM (Gavazzo et al., 2008). Similar findings were published by Lasley and Gilbert (1999) using cortical neurons from adult rats. The IC_{50} for Pb^{2+} ranged from 1.52 to 4.86 µM, with the ranking of Pb^{2+} potency in inhibition of NMDAR subunits to be NR1b-2A>NR1b-2C>NR1b-2D>NR1b-2AC after experiments that have been conducted in Xenopus oocytes injected with cRNAs for different combinations of NMDA receptor subunits (Omelchenko et al., 1997). Gavazzo P, Zanardi I, Baranowska-Bosiacka I, et al (2008) Molecular determinants of Pb^{2+} interaction with NMDA receptor channels. Neurochem Int 52(1-2): 329-337.*

Comment-4: The reviewers suggested that Pb^{2+} does not always act as an NMDAR inhibitor but it can also behave as an NMDAR activator under certain conditions ("Uncertainties and inconsistencies", Page 26).

Response: *Pb^{2+} can act as an NMDAR inhibitor or activator under certain circumstances. This aspect is already addressed in KER: KEup Binding of antagonist, NMDAR to KEdown inhibition, NMDARs, in the section Uncertainties. During the TC, the reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

Comment-5: The reviewers requested clarification of "Pb^{2+} at 30 µM stimulates calmodulin activity, but inhibits at higher concentration" (Page 28) (Sandhir and Gill, 1994).

Response: *The authors indicated that a sentence will be added in empirical support of KER NMDA inhibition leads to decreased Ca^{2+} influx: Calmodulin, as a Ca^{2+} binding protein, has been strongly implicated in Pb^{2+} toxicity. However, interactions between Ca^{2+} and Pb^{2+} on several binding sites suggest complex interactions (Kirberger, 2011). Kirberger, M. (2011) Defining a molecular mechanism for lead toxicity via calcium-binding proteins. Chemistry dissertation. Georgia State University. ScholarWorks@Georgia State University.*

Comment-6: The reviewers suggested supplementing information on interactions between the other heavy metals (as discussed on page 18) and NMDARs.

Response*: The authors decided to delete the paragraph.*

Comment-7: The reviewers suggested introducing a KE indicative of cellular and organ events leading to learning and memory impairments in aging. This KE would represent a point of conjunction of different stressors able to induce learning and memory disabilities.

Response: *According to the principle of AOP, KE is independent of life stage applicability. The information about life stage applicability is specified separately. This was done in order to keep the KE description general and to allow re-use of KEs in other AOPs. During the TC, the reviewers agreed that the responses provided by the authors were adequate and no further actions are deemed necessary.*

Comment-8: The reviewers recognized the importance of KE "Neuroinflammation" due to its involvement in several neurodegenerative processes, but neuroinflammation can also be involved in neuroprotective/neuroreparative properties when activated. The reviewers, however, raised a concern about the positioning of this KE by stating that if neuroinflammation was demonstrated to participate in protein aggregation and tau hyperphosphorylation, and neurodegeneration in AD, its implication in the reduction of the neuronal network activity and learning and memory dysfunction independently from AD is questioned and this may not be a KE in such a case. The reviewers further reiterated positioning of KE "Neuroinflammation", which may depend on the objective of the AOP and this appears to be a bit ambiguous. The reviewers further requested clarification as to whether the objective of this AOP is to assess a potent long-term cognitive impairment possibly related to an early exposure to a glutamate-NMDAR interacting compound or to assess the more specific risk of having AD as a consequence of such exposure? In the latter

case, neuroinflammation may not have to be considered as a KE. But if the objective is modeling of the neurotoxicity of substances leading to AD, neuroinflammation has to be positioned as a KE. The authors were requested to provide clarification.

***Responses**: The question of inflammation in the different organs and of neuroinflammation in the brain has been very much debated in the AOP. One of the authors belongs to a group in OECD and the authors will have a workshop in September 2017 in order to discuss if they have to consider inflammation as a real KE or as a modulatory factor, and what would be the best way to represent it in an AOP. The authors already had this discussion during the preparation of AOP- 3. Because there is so much literature showing that inhibition of any feature of neuroinflammation can have protective effects on DA neurons or terminals degeneration, they decided to consider it as a real KE. Therefore, before changing anything to KE neuroinflammation, the authors prefer to wait for the results of this September workshop. As AOPs are living documents, changes can be applied later on, even when AOP is endorsed.*

Furthermore, at the time of TC discussion, the authors were requested to include that neuroinflammation can be a cause or a consequence of neurodegeneration. This was already present in the KER neurodegeneration leads to neuroinflammation, but the text was completed according to reviewers' suggestion. The fact that neuronal death can trigger neuroinflammation and that neuroinflammation can, in turn, cause neuronal degeneration, is known as a vicious cycle, which is involved in the pathogenesis of neurodegenerative diseases (Griffin et al., 1998; McGeer and Mc Geer, 1998; Blasko et al., 2004; Cacquevel et al., 2004; Barbeito et al., 2010; Rubio-Perez and Morillas-Ruiz, 2012; Thundyil and Lim, 2015).

Microglial cells are involved in the clearance of amyloid plaques (Querfurth and LaFerla, 2010). But microglia can also be responsible for amyloid plaque formation (Streit and Sparks, 1997). As aging microglia seem to lose their ability of phagocytosis (Floden and Combs, 2011), a default in clearance as well as active deposition can both contribute to amyloid plaque accumulation.

Comment-9: In the context of the role of neuroinflammation in neurodegenerative disorders as well as in neuroprotective processes, the reviewers noted that the authors have given more emphasis to microglial cells, and less to astrocytes. The authors need to provide more details on astrocytes because of their importance in the regulation of the glutamate neurotransmitting concentration and the NMDAR activation.

***Response:** The authors agree with the reviewers and have accordingly stated that much less is known for reactive astrocytes in the context of neuroinflammation than for reactive microglia. Recently, Liddelow et al (2017) described an analogy to microglial phenotypes a A1 neurotoxic reactive astrocyte phenotype. The authors also included the measure of C3 as a marker of this neurotoxic reactive astrocyte phenotype. This information has been added in the KE Neuroinflammation. During the TC discussion, the reviewers agreed that the responses and actions provided by the authors were adequate and no further actions are deemed necessary.*

Comment-10: The reviewers suggested that an increase in the glial fibrillary acidic protein (GFAP) expression does not mean astrogliosis, but increased astrocytogenesis from the neural stem cells, and therefore, it should be revised accordingly (Page 35).

Response: In KER Cell injury leads to neuroinflammation, the authors deleted the reference of Chan *et al* (2013) about neural stem cells treated with Pb^{2+}, in order to avoid any confusion between Pb^{2+}-induced astrocyte differentiation and real astrogliosis observed in already differentiated astrocytes.

Comment-11: The reviewers identified Pb^{2+} as a glutamate-NMDAR modulator rather than as a pharmacological antagonist, and compared it with pharmacological drugs that are NMDAR antagonists successfully used in the treatment of AD. The authors were requested to clarify this contradiction on a mechanistic basis.

Response*: Per discussion during the TC, the authors added the following sentences in Overall assessment of the AOP, in chapter 2. Strength, consistency and association of AO with MIE: Interestingly, memantine, an NMDAR antagonist used in the treatment of AD, was shown to improve cognitive functions (for details, see Dekundy, 2006). This might seem in contradiction with the described AOP considering* Pb^{2+} *as an antagonist of NMDAR and its potential risk to cause cognitive deficits and amyloid plaque accumulation, which are hallmarks of AD. However, the window of exposure differs completely, since memantine is applied in aged patients when the disease has broken out; whereas the risk of delayed neurodegeneration described in this AOP is due to NMDAR inhibition during brain development.*

Comment-12: Since it is recognized that extra-synaptic NMDARs is the shut off signal of CREB (Page 30), the reviewers suggested supplementing this information with the functions of NMDARs, which depends on the cellular localization (synaptic or extra synaptic) (Hardingham *et al*., 2002, *Nat Neurosci* **5**: 405-414).

Response: *The authors will add the following sentences in KER decreased* Ca^{2+} *leads to decreased BDNF release. Interestingly, synaptic and extra-synaptic NMDARs have opposite effects on CREB.* Ca^{2+} *entry through synaptic NMDAR induces CREB activity and BDNF gene expression. In contrast,* Ca^{2+} *entry through extra-synaptic NMDAR activates a general and dominant CREB shut-off pathway that blocked induction of BDNF expression (Hardingham GE and Bading H. 2002. Coupling of extrasynaptic NMDARs to a CREB shut-off pathway is developmentally regulated. Biochim Biophys Acta 1600(1-2): 148-153).*

Comment-13: The reviewers agreed with the authors that there are some data showing a link between exposure to Pb^{2+} in early life to learning impairment in later life, and the timing of the exposure to Pb^{2+} might influence AD pathogenesis. The reviewers, however, wanted to know whether this AOP is active only during this time frame or chronically active until the onset of the memory impairment. The reviewers proposed adding another KE, such as "Progression of

neurodegeneration" or "Progression of neuroinflammation" to explain the delay of AO (Page 43).

Response: *The authors stated that the proposed AOP is based on the experimental studies showing that an early exposure leads to delayed effects. Clearly, progression of neurodegeneration and of neuroinflammation is involved in this process. The progressive aspect is illustrated by the "turning arrows" linking neuroinflammation and neurodegeneration, meaning that there is a vicious cycle between these 2 KEs and when neurodegeneration is sufficient, then it goes to the next KE, impairment of learning and memory. According to the concept of AOP, for the re-use of KEs in several AOPs, the description should be as basic as possible and supplementary information about duration and life stage applicability are added in the text. During the TC, the reviewers agreed that the responses provided by the authors were adequate and no further actions are deemed necessary.*

Comment-14: The reviewers sought more details from authors regarding the hippocampus-dependent tasks in rodents, monkeys and other animal species where the AOP discusses the effects of developmental neurotoxicants on AD-related learning and memory impairments, which are based on deleterious effects on hippocampal networks.

Response: *The authors asserted that the best characterized form of long-term potentiation (LTP) occurs in the CA1 sector of the hippocampus, in which LTP is initiated by transient activation of receptors and is expressed as a persistent increase in synaptic transmission through AMPA receptors followed by activation of NMDARs. This increase is due, at least in part, to a postsynaptic modification of AMPA-receptor function; this modification could be caused by an increase in the number of receptors, their open probability, their kinetics or their single-channel conductance. Summing up activity-dependent alteration in synaptic strength is a fundamental property of the vertebrate central nervous system that underlies learning and memory processes.*

During the TC, it was discussed whether the hippocampus is solely involved in learning and memory or if other regions of the brain are involved as well. The authors stated that while much emphasis has been given to the key role of the hippocampus in memory, it would probably be simplistic to attribute memory deficits solely to hippocampal damage (Barker and Warburton, 2011). There is substantial evidence that fundamental memory functions are not mediated by hippocampus alone but require a network that includes anterior thalamic nuclei, mammillary bodies cortex, cerebellum and basal ganglia, in addition to hippocampus (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005). Each of these brain structures can potentially be damaged leading to more or less severe impairment of learning and memory.

Furthermore, in the past, the study of infant memory has relied on models and tests used in adults and more specifically amnesic patients with hippocampal damage. For this reason, the infant memory has been distinguished as declarative or explicit memory and nondeclarative or

implicit memory. However, in recent years, the distinctions of explicit/implicit are no longer accepted, especially in relation to hippocampal function, as new theories have emerged (reviewed in Mullally and Maguire, 2014). Furthermore, there are findings that even very young infants have a more adept and flexible memory system than was previously thought and neurobiological data derived from non-humans provided support to the new hypotheses about hippocampal development that would facilitate interpreting infant memory data from humans.

During the TC discussion, the authors provided additional information on hippocampus-specific tasks that is now introduced in KE learning and memory impairment. The reviewers proposed to send the additional informations, and that will be included in the revised AOPWiki.

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

Comment-1: The reviewers agreed with authors that the markers of neuroinflammation are not well established in regulatory toxicity studies. This AOP may provide an impetus to increase inclusion of markers of neuroinflammation in regulatory studies, since the AOP provides information that shows a link between neuroinflammation and neurodegeneration.

Response: *The authors are convinced that even if neuroinflammation is a late KE, it can be tested using an in vitro 3D culture system containing all main neural cell types, but at the minimum neurons, astrocytes and microglial cells. The 3D rat brain cell culture system that the authors have used for neurotoxicological studies for about 30 years is adequate for testing neuroinflammation, and new development with human stem cells will also soon be allowed for such testing. The question of high throughput screening remains a problem to be solved. During the TC discussion, the reviewers agreed that the responses provided by the authors were adequate and no further actions are deemed necessary.*

Comment-2: The reviewers indicated that this AOP can be used in developmental neurotoxicity as well as for hazard identification of compounds that interact with NMDARs, and the unraveling of mechanisms underlying the occurrence of neurological disorders like neurodegeneration in later life. The authors cautioned, however, that the WOE must be accurate because the final AO is severe and has a great impact.

Response: *During the TC discussion, the authors asserted that they are aware of the fact that this AOP describes only a single risk (among many others) of developing a neurodegenerative disease such as AD. As neuroinflammation and neurodegeneration are two late KEs that are common to several AOPs and that are the consequences of several distinct MIEs, the authors think that the networking of several AOPs (as proposed in AOP Wiki) will give a more accurate overview of the biological complexity of such a disease.*

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

Comment-1: All reviewers agreed that this AOP is well written and the contents are supported by peer-reviewed scientific literature. The conclusions regarding the strength of the scientific data that support the KEs and KERs are reasonable.

Response: *The authors greatly acknowledged the reviewers' compliments.*

Comment-2: The reviewers noted a weakness that this AOP is entirely defined by a single environmental chemical, i.e. Pb^{2+}. AOPs are not supposed to be chemical specific. Is there any evidence that other NMDAR antagonists result in similar effects on KEs?

Response: *During the TC discussion, the authors agreed to add the following paragraph in Overall Assessment of AOP, chapter 2. Strength, consistency and association of AO and MIE. This AOP is defined by a single environmental chemical, i.e* Pb^{2+}*. However, other NMDAR antagonists, such as MK 801, phenylcyclidine, and ketamine, when applied during brain development may also lead to functional impairments in cognitive domains relevant to memory. The effects of these drugs on brain function appear to have a delayed onset, and can be very long-lasting if not permanent. In general, longer durations, higher concentrations and longer or repeated exposures tend to exacerbate impairments (for review, see Walters and Paule, 2017). The mechanisms underlying these NMDAR antagonists-induced neurotoxicity are unclear, but several hypotheses have been proposed: impairment of mitochondrial integrity and function, dysregulation of intracellular calcium and neuroinflammation have all been implicated (Lei et al., 2012). Some of these mechanisms are common to the KEs described in this AOP, suggesting that such delayed effects on memory processes can be a general consequence of developmental brain exposure to NMDAR inhibitors. However, no studies have yet reported that these other NMDAR inhibitors cause amyloid plaque deposition and tau hyperphosphorylation associated with AD like neurodegeneration when aging.*

Comment-3: The reviewers suggested that a new KE should be added, which is indicative of cellular and organ events leading to learning and memory impairments in aging.

Response: *During the TC discussion, the authors stated that an AOP is a simplification of a complex biological process. According to AOP principle, KEs are critical events, which when disturbed enough lead to the next step/KE. Therefore, plasticity, adaptive or compensatory mechanisms in the network of memory cannot be captured as a critical KE. However, such information can be added in the description of the KE impairment of learning and memory when the authors receive such information from the reviewers.*

Comment-4: The reviewers requested the authors to add information on various scenarios of Pb^{2+} exposure with modulation of the duration and the level of exposure that can be helpful for validation of this AOP.

Response: *The paper by Bihaqi et al (2014) described delayed cognitive impairment only if animals have been treated when young. However, the proposed paper by the reviewers*

(Schwartz et al., 2000) has associated delayed cognitive decline with former Pb^{2+} workers, who were exposed during adulthood. During the TC discussion, the reviewers offered to provide information about various scenarios of Pb^{2+} exposure in humans leading to delayed cognitive deficits, which will be added in Overall assessment, in section 1. Concordance of dose-response and temporal concordance.

Comment-5: The reviewers cited a scenario where the levels of Pb^{2+} exposure and blood Pb^{2+} concentrations seem to be quite different between Pb^{2+} - inducing AD disturbances and cognitive impairments in children. This point in the AOP was weak, and therefore detailed information in the overall assessment part of the AOP was requested to be supplemented.

Response: *During the TC, the issue was discussed, and accordingly, the authors added the text in Overall assessment, chapter Quantitative considerations. With an Adverse Outcome occurring after such a long delay following the MIE, it is extremely difficult to make a quantitative link, since the AO can occur when serum Pb^{2+} levels have returned to normal. Bakulski and coworkers (2012) therefore proposed measuring Pb^{2+} bone content as an index of historical Pb^{2+} exposure. Similarly, Schwartz and coworkers (2000) showed that tibia Pb^{2+} levels were good predictors of delayed cognitive decline of former organolead workers. Thus, Pb^{2+} blood level is rather representative of acute exposure, whereas Pb^{2+} bone level represents a long-term accumulation.*

Comment-6: In the overall assessment part of the AOP, the authors described studies assessing the effects of occupational Pb^{2+} exposure on neuropsychological performances of workers, and present epidemiological evidence for association of Pb^{2+} with AD. These studies indicate that: 1) bone Pb^{2+} levels are indicative of cumulative Pb^{2+} exposure, whereas blood Pb^{2+} concentrations are more relevant to acute exposure, and 2) that correlations between bone Pb^{2+} levels and neuropsychological disturbances were found to disrupt more locomotor coordination abilities and executive functions than cognitive performances. The reviewers cautioned the authors for referring to these studies because: 1) the Pb^{2+} exposure concerned only the adult stage and did not occur during the early life of people, and 2) cognitive performances of Pb^{2+} -exposed workers remained unaffected.

Response: *The authors removed the references about the locomotor coordination and modified the table of essentiality for the AO at the organism level: Impairment of learning and memory. The authors introduced a paper here proposed by the reviewers showing a delayed decline of cognitive functions occurring long after Pb^{2+} exposure has ceased. The lack of epidemiological evidence linking early Pb^{2+} exposure and late neurodegeneration of AD type is more clearly explained, but the strong experimental evidence and the principle of delayed effects have been proposed as being sufficient to consider such early exposure as a risk factor for neurodegeneration. During the TC discussion, the reviewers agreed with authors on this action. However, any additional information provided by the reviewers will be added to better illustrate this aspect in the Overall assessment.*

Comment-7: The reviewers have acknowledged the importance and usefulness of this AOP for regulatory purposes.

Response: *During the TC, the authors thanked the reviewers for their compliments.*

Minor issues: The reviewers highlighted a few minor issues and mistakes that needed the attention of the authors:

Comment-1: The reviewers noted several inconsistencies in grammar and use of the English language, and therefore suggested editing by a English-language proficient editor.

Response: *Rex FitzGerald, who is co-author and a native English speaker, reviewed the AOP before its implementation in AOP Wiki.*

Comment-2: Page 43, line 3 under the heading "Uncertainties or Inconsistencies".
It should be 'lifetime', and not 'leftime',

Response: *Typing error has been corrected.*

Comment-3: Page 46, line 3: Avoid repetition of 'of'.

Response*: Typing error has been corrected.*

Summary record of the teleconference

A teleconference (TC), using WebEx, was held on May 17, 2017, and the participants were the authors (Florianne Tschudi-Monet and Rex FitzGerald), the reviewers (April Kluever, Hester Hendriks, Kaoru Sato, and Henri Schroeder) and the OECD administrator Magdalini Sachana. External review manager (Ramesh Gupta) served as moderator to facilitate the TC process. During the TC, main issues were discussed point by point until both the reviewers and the authors were satisfied and all issues were resolved. It was noteworthy that the reviewers graciously offered additional information to the authors to resolve the main issues to further enhance the quality of this AOP. The authors responded to each issue and all those responses are reflected in the AOPWiki. From the administrator's perspective, Magdalini Sachana was very helpful in explaining the policies and procedures of the review process, as and when requested by the reviewers and the review manager. Overall, the outcome of the TC was very constructive, productive and cordial. By the end of the TC, all main issues were resolved. During the TC, it was discussed and decided that each issue was from all the reviewers and the response was from both authors to avoid redundancy.

Summary of revisions

The authors responded to the majority of the reviewers' queries prior to the TC, and to all queries during the TC. The discussion and explanations are reflected in the AOPWiki. Of course, the

authors stated that the AOPWiki is a living document and it can be amended at any time as additional information becomes available.

Further discussion

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

Outcome of the external review

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

Concluding remarks

As review manager, I would like to thank the authors/developers of this excellent AOP on such a timely topic that interests not only toxicologists in academia, industry and regulatory sectors, but many in other disciplines as well. The authors proposed a pathway, i.e., chronic binding of Pb^{2+} to NMDARs during the early brain developmental stage linking it with a neurodegenerative disease such as Alzheimer's in later life. The painstaking efforts of the highly qualified scientists who served as the reviewers are noteworthy. They devoted their time in reviewing the AOP draft, identifying the deficiencies and main issues and participating in the teleconference (TC) held on May 17, 2017. During the TC, each issue was discussed and the authors provided adequate response, discussion and explanation, which are described in this report and are reflected in the revised AOPWiki. During the TC, the reviewers also offered the authors additional information so as to enhance the scientific quality of this AOP. All reviewers were satisfied with authors' responses. The authors appreciated the reviewers immensely for their input to this AOP. Finally, I would like to thank the OECD administrators (Nathalie Delrue and Magdalini Sachana) who offered their assistance to the reviewers, authors and the review manager at every step of the review process.

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