Organisation for Economic Co-operation and Development

Adverse Outcome Pathway External Review Report

AOP 10 Binding to the picrotoxin site of ionotropic GABA receptors leading to epileptic seizures in adult brain

Short title: Blocking iGABA receptor ion channel leading to seizures

The title of the AOP was revised during the review.

Original Title: Binding to the picrotoxin site of ionotropic GABA receptors leading to epileptic seizures

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1. Introduction and background to specific AOP

Ionotropic GABA receptors (iGABARs) are ligand-gated ion channels, which play important functional roles in the nervous system. As the major player in inhibitory neurotransmission, iGABARs are widely distributed in both vertebrates and invertebrates. In vertebrates, the iGABAR includes two subclasses of fast-responding ion channels, $GABA_A$ receptor ($GABA_A$-R) and $GABA_C$ receptor ($GABA_C$-R). Invertebrate iGABARs do not readily fit the vertebrate $GABA_A$/$GABA_C$ receptor categories. The majority of insect iGABARs are distinguished from vertebrate $GABA_A$ receptors by their insensitivity to bicuculline and differ from $GABA_C$-Rs in that they are subject to allosteric modulation, albeit weakly, by benzodiazepines and barbiturates.

Chemical interactions with iGABARs can cause a variety of pharmacological and neurotoxicological effects depending on the location of the active or allosteric site affected. Three distinct types of interactions at binding sites on iGABARs can antagonize the postsynaptic inhibitory functions of GABA and lead to epileptic seizures and death. These three types of interactions correspond to three distinct AOPs. One of the three types of interaction is non-competitive channel blocking at the picrotoxin convulsant site located inside of the iGABAR pore that spans neuronal cell membranes (this MIE) and it has been developed by the U.S. Army Corps of Engineers. The other two types of interactions are negative modulation at allosteric sites and competitive binding at the active orthosteric sites (MIEs to be developed in the future).

This AOP named “Binding to the picrotoxin site of ionotropic GABA receptors leading to epileptic seizures in adult brain”begins where chloride conductance through the ion channel is blocked due to chemical binding at or near the central pore of the receptor complex (i.e., the picrotoxin site). As a result, the first key event (KE) is a decrease in inward chloride conductance through the ligand-gated ion channel. This leads to the second KE, a reduction in postsynaptic inhibition, reflected as reduced frequency and amplitude of spontaneous inhibitory postsynaptic current (sIPSC) or abolishment of GABA-induced firing action in GABAergic neuronal membranes. Consequently, the resistance of excitatory neurons to fire is decreased, resulting in the generation of a large excitatory postsynaptic potential (EPSP), i.e., the third KE. The large EPSP is reflected as a spike (rise) of intracellular Ca^{2+} observed in the affected region, where a large group of excitatory neurons begin firing in an abnormal, excessive, and synchronized manner. Such a giant Ca^{2+}-mediated excitatory firing (depolarization) causes voltage-gated Na^{+} to open, which results in action potentials. The depolarization is followed by a period of hyper-polarization mediated by Ca^{2+}-dependent K^{+} channels or GABA-activated Cl^{-} influx. During seizure development, the post-depolarlization hyperpolarization becomes smaller, gradually disappears, and is replaced by a depolarization. This characteristic depolarization-shrinking hyperpolarization sequence of events represents the fourth KE known as “paroxysmal depolarizing shift” (PDS), which forms a “seizure focus”. A PDS is, essentially, an indication of epilepsy at the cellular level, which serves as the foci to initiate the adverse outcome at the organismal level of epileptic seizure. The severity of

symptoms is often dose- and duration- dependent, while the toxicological symptoms are associated with the type and location of affected iGABARs. Mortality can occur if the individual sustains a prolonged or pronounced convulsion or seizure. Neurotoxicity, of which seizures are an end point, is a regulated outcome for chemicals. This AOP allows for screening chemicals for the potential to cause neurotoxicity through the use of in vitro assays that demonstrate binding to the picrotoxin site, electrophysiological assays demonstrating depolarization of neuronal membranes, or electroencephalography that records electrical activity of the adult brain.

2. Synthesis of main issues of the review

This section provides an overview of issues raised by 5 external reviewers (reviewers' details in Annex 1) of AOP 10 in their answers to four charged questions given (i.e. scientific quality, weight of evidence, regulatory applicability and overall assessment). Each reviewer's individual comments and answers to charged questions are provided in Annex 2.

1. Scientific Quality

- **Need for additional information:** Many reviewers felt that the bibliography provided in the AOP was insufficient in certain sections, especially bibliography related to the Key Events (KEs) and the methods used to measure the KEs. Some reviewers also suggested expanding the stressors lists for the Molecular Initiating Event (MIE) and Key Events Relationships (KERs).
- **Scope of the AOP:** The domain of applicability of this AOP was not clear to some reviewers on whether the AOP can be applied to brains of developing organisms or not. In addition, a reviewer also suggested changing the title from "leading to epileptic seizures" to "leading to convulsive seizures" to specify the scope.
- **Need for further clarification:** Reviewers requested for clarification in few parts of the AOP, e.g. clarification of the three subsequent steps outlined in the Adverse Outcome (AO) as well as clarification of the sequence of events leading to paroxysmal depolarizing shift.

2. Weight of Evidence (WOE)

In general, the reviewers agreed to the WOE scoring provided in the AOP. No significant issues were raised, however it was noted that pending the suggestions to the scientific quality, the WOE scoring might have to be reconsidered.

3. Regulatory Applicability

The reviewers generally agreed with the authors' statement that this AOP can potentially be used to establish mode of neurotoxicological actions for the chemicals capable of binding to the picrotoxin convulsant site of iGABARs as well as to assist in predictive modeling of chemical toxicity in risk assessment. In the meantime, the reviewers concurred that it is too early to judge the regulatory use of the AOP. As the methodologies for hazard and risk assessment of chemicals and drugs are likely to evolve and be based on more *in vitro* and *in silico* approaches, the importance of this AOP for regulatory purpose may increase in the future. Furthermore, as mentioned above, the domain of applicability should be well clarified that this AOP is not applicable to developing organisms.

4. Overall Assessment

The reviewers acknowledged that this AOP is well constructed. Nonetheless, the following concerns remained to be addressed:

- Domain of applicability: life stage and brain structure. Do the authors need to expand and clarify further the domain of applicability?
- Is there need to explain the mode of action for seizures occurring in young children?
- Is there a need to better capture the limitations of this AOP?
- Do the data gaps need to be better discussed?

3. Summary record of the teleconference

3.1. TC agenda (TC 12 Feb 2018, 14:00 CET)

1. Confirm agenda items with attendees. Any other items?

2. Issues resulting from Charge question 1.

Scientific quality:

- Does the AOP incorporate the appropriate scientific literature?
- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

2.1. Need for additional bibliography related to KEs, including methods that are used to measure KEs.

2.2. Need for additional stressors for the MIE and KERs (empirical support).

2.3. Merging KEs: "decreased Cl-conductance" and "reduced neuronal synaptic inhibition".

2.4. Redefining the AO and changing the title of the AOP: substituting "leading to epileptic seizures" to "leading to convulsive seizures". Could this change address some of the concerns related to the applicability domain of the AOP?

2.5. Clarifying the three subsequent steps outlined in the AO (increased K^+, accumulation of Ca^{2+}, and activation of NMDA-Rs). Why are these not considered 3 separate KEs? If you prevent Ca^{2+} accumulation or NMDAR activation, would that block the seizure? Are these mechanisms operating simultaneously or separately? Is it suggested that during block of the GABAergic channel by picrotoxin; somehow it can be reactivated by binding of GABA leading to Cl^- influx? Are there evidence supporting that?

2.6. Clarifying the sequence of events leading to paroxysmal depolarizing shift.

2.7. Need for additional narrative. This may be covered above but need to check.

3. Issues resulting from Charge question 2. Weight of evidence scoring.

3.1. Scoring of the KERs does not seem to have generated issues, pending the revisions suggested above. Should the authors have to reconsider the scoring after adding more stressors and relevant bibliography?

4. Issues resulting from Charge question 3. Regulatory applicability.

4.1. All of the reviewers concur that it is too early to judge what the regulatory use of the AOP. In the future, as the methodologies for hazard and risk assessment of chemicals and drugs are likely to evolve and be based on more in vitro and in silico approaches the importance of this AOP for regulatory purpose may increase.

5. Issues resulting from Charge question 4. Overall assessment.

Domain of applicability: life stage and brain structure. Do the authors need to expand and clarify further the domain of applicability? Is there need to explain the MOA for seizures occurring in young children? Is the AOP applicable to specific brain area? Is there a need to better capture the limitations of this AOP? Do the data gaps need to be better discussed?

6. Other issues identified by reviewer/authors.

7. Next steps.

3.2. Main issues and responses during the call

The main discussion revolved around the 3 issues under scientific quality in Section 2: Synthesis of main issues of the review. In short, following points were discussed and agreed:

- The reviewers offered to provide references for several sections in the AOP. The authors will then include the suggested references to have sufficient bibliography of the AOP.
- To clarify the scope of the AOP, the title of the AOP will include the wording "adult brains".
- Authors will add more details to elaborate sufficiently in the parts which lack clarity.

Detailed action plans of the teleconference are listed in the section below.

3.3. Action list

Action Points for Reviewers

- R1 will provide reference on methodology regarding Event 669: Reduction, Neuronal synaptic inhibition to the authors.
- RM will distribute the Word document of the AOP and R3 will indicate editorial issues (e.g. typos, grammatical errors, abbreviations) needed to the authors.
- R3 will provide bibliography on convulsions in human/mammals to the authors. The authors will then use the information to update relevant Sections.
- R5 will provide reference on the role of other cations such as intracellular Mg^{2+} and by extension ATP levels and phosphorylation of $GABA_A$ channels plays in ion channel conductance leading to epileptic seizures to the authors.

- RM will highlight the parts in Users' Handbook to the Authors, which explain about what information a KE description should contain and how to add chemical stressors' information in the empirical evidence section of KERs . Authors will then revise the KEs and KERs accordingly.

Action Points for Authors

Title:

- Authors will add "adult brain" in the title of AOP to clarify the domain of applicability (life stage) to this AOP.

Abstract:

- Authors will revise the Abstract of AOP on the following part: "…the period of hyperpolarization can then be mediated by either Ca^{2+}-dependent K^{+} channels or GABA-activated Cl^{-} influx."

MIE (Event 667: Binding at picrotoxin site, iGABAR chloride channel):

- Authors will add information in MIE to clarify that the MIE is relevant to adult brains only (R1).
- Authors will add more information on description of MIE (R2).
- Authors will add information on GABAb receptors in the uncertainty part of MIE (R5).
- Authors will provide more details on biological activity of the binding site (R4).

KEs:

- Authors will revise the KEs table and use the new AOP wiki to put in the right order the KEs as they appear in the AOP diagram (R3).

KE#1(Event 64: Reduction, Ionotropic GABA receptor chloride channel conductance):

- Authors will add more information to elaborate further on the methodology (R4).

KE#2 (Event 616: Occurrence, A paroxysmal depolarizing shift):

- Authors will provide response to R4's comment and send it to the RM (R4).

KE#3 (Event 669: Reduction, Neuronal synaptic inhibition)

- Authors will check if occurs in the cellular/tissue level and modify the KE accordingly (R3).

KE#4 (Event 682: Generation, Amplified excitatory postsynaptic potential (EPSP)):

- Authors will elaborate on the methodology based on the comment by reviewer (R1).

AO:

- Authors will double check the literature regarding 3 subsequent steps outlined in AO and clarify any uncertainties in these steps.

Weight of Evidence:

- Authors will check if any new evidence (e.g. new stressors' information) added to the AOP would affect the scoring and revise accordingly.
- Authors will add more details to explain the quantification of the WOE that was applied and check the compatibility with Users' Handbook.

4. Summary of planned revisions

All planned revisions are based on the teleconference which took place in 12 Feb 2018 and the details are provided in Section 3.3 Action list.

5. Further discussion

No further discussion was required as all issues were resolved in the teleconference.

6. Outcome of the external review

All the reviewers of this AOP felt that the authors did an excellent job in drafting the AOP entitled "Binding to the picrotoxin site of ionotropic GABA receptors leading to epileptic seizures in adult brain" and acknowledged the significant contribution of the authors through the development of this AOP to the AOP-Knowledgebase. All the reviewers appreciated the amount of work that has gone into this AOP that hopefully will stimulate further research in this field and its application in the regulatory arena. The reviewers devoted significant amount of their time to provide constructive comments, editorial changes and additional literature and all these materials have been made available to the authors. The issues raised from the reviewing process and discussed in the conference call, received the support from all the members of the review panel and the authors agreed to implement the suggested changes by updating and making changes to specific sections of this AOP.

All the reviewers of this AOP felt that once the changes are implemented, it will be appropriate that this AOP gets approval and eventually published. There was agreement that the AOP could be considered very useful for regulatory purposes.

Although the authors haven't revised fully the AOP to reflect the reviewers' comments at the time this report is drafted, the authors indicated to the review managers that they are willing to do so by the end of May 2018, so that the AOP can be considered for EAGMST approval at the June meeting. The authors provided replies to the comments on the original charge questions prior to the teleconference but the authors will need to further explain how the actions arising from the teleconference will be addressed and store this information in the AOP-Wiki discussion pages.

Annex 1: Table with reviewers' name

Reviewer	Name	Country/Affiliation	Contacts
Review Manager	Magda Sachana	OECD	Magdalini.SACHANA@oecd.org
Review Manager	Ye Lin Jun	OECD	YeLin.JUN@oecd.org
Reviewer	Cristina Sunol	Spain	cristina.sunol@iibb.csic.es
Reviewer	Frank Johnson	United States	Frank.Johnson@fda.hhs.gov
Reviewer	Gerrit Wolterink	Netherlands	gerrit.wolterink@rivm.nl
Reviewer	Timothy Shafer	United States	Shafer.Tim@epa.gov
Reviewer	Yasunari Kanda	Japan	kanda@nihs.go.jp

Annex 2: Individual reviewers' comments and written response from the authors in preparation for the end of review Teleconference

Reviewer 1 (R1)

Scientific Quality

- Overall, too terse and needing more documentation.

Under life stage for KE#1. There is plenty of information that activation of GABA receptors in the embryonic/early postnatal (through PND~7), activation of GABA-A receptors can be excitatory, rather than inhibitory. Thus, this key difference in life stage may have profound influence on the downstream KEs and the Adverse Outcome. This needs to be acknowledged and discussed.

Response: Need to clarify that KE#1 refers to "Reduction in Ionotropic GABA receptor chloride channel conductance" (Event ID 64), not MIE, because KE #s in the following comments apparently correspond to sequence #s. Although this comment is valid, we have already addressed it in the "Domain of Applicability" on the AOP's main page: "This AOP may not be applicable to young animals during their embryonic and early developmental stages when GABA acts as an excitatory neurotransmitter due to increased intracellular Cl^- concentration in inmature or developing neurons (Taketo and Yoshioka 2000; Galanopoulou 2008)….". As pointed out by the reviewer, such a developmental biology problem not only affects KE#1 but may cause cascading effects on downstream KEs and the AO. Therefore, we chose to discuss this issue at the AOP level, instead of the KE level.

Under "how it is measured" for KE#1. Chloride-sensitive dyes can also be used to detect changes in function of GABA receptors. See work by Jon Inglefield and Rochelle Schwartz-Bloom, among others.

Response: We have added this method (and reference) as suggested.

The "Domain of Applicability" section for KE#1 is also a bit thin. There are numerous studies in different preparations where modulation of GABA receptors is demonstrated by organochlorine insecticides and other compounds. See work by Narahashi, for example. There are many others.

Response: We have noticed the work by T. Narahashi which covered not only the GABA system but also the sodium channel modulators (see Narahashi T. 1996. Pharmacol Toxicol. 79(1):1-14). Hence, we did not include this reference previously. In recognition

of this reviewer's comment, we have cited this reference and added most organochlorine and some pyrethroid compounds that are proven insecticides sharing the same mode of action through blocking the chloride channel.

Key event #4, reduction, neuronal synaptic inhibition.

The Key event description here and how it is measured are both very terse. GABA-A inhibition and resultant seizures are a widely-studied phenomenon in the central nervous system. While I am unable to point to specific reviews, there is much more information available than is presented here. It is incumbent on the authors of the AOP to be more thorough in their presentation.

Response: Indeed there are numerous studies and reviews on $GABA_A$ inhibition and resultant seizures. We have cited many of them throughout this AOP. However, for this particular KE (Event ID 669), we provided a precise and concise presentation. Therefore, we respectfully disagree with this reviewer on the characterization of our presentation being terse.

Key Event #5. Again, the how it is Measured section is too terse. IPSP are not even mentioned. The Domain of applicability is also not adequately detailed. There are also numerous studies on rats and other species that should be considered.

Response: It is true that we did not provide details on any measure method throughout this AOP. Our main consideration is that it would be better for one to read, interpret and set up the referred methods, instead of following our description and interpretation. In addition, we ourselves don't have first-hand working experience with all of the referred methods. As for IPSP, it is not the endpoint this KE measures. Therefore, we did not provide any measurement method for IPSP.

In the description for KE#6 (the Adverse Outcome), three subsequent steps are outlined (increased K+, accumulation of Ca, and activation of NMDA-Rs). Why are these not considered 3 separate Key Events? If you prevent Ca accumulation or NMDA activation, would that block the seizure?

Response: These three events are intermediated and transient. They are also intertwined and can't be separated, jointly leading to seizure propagation. Therefore, they cannot be considered as KEs.

Under Stressors, only Picrotoxin, Lindane and Dieldrin are listed. This is incomplete. Several other organochlorine insecticides interact with the GABA receptor in a manner similar to lindane and dieldrin (eg Heptachlor, endosulfan, toxaphene, etc) as well as RDX, Fipronil should also be considered.

Response: We have added more chemical stressors, as suggested by the reviewer, including heptachlor, endosulfan, RDX and fipronil. We did not add toxaphene because it is a mixture of over 670 chemicals. In addition, we'd like to acknowledge that it would be a community-wide endeavor to make an exhaustive list of all chemical stressors sharing this mode of action as described in AOP10.

Weight of Evidence

Overall, I agree with the WOE scoring. However, due to the lack of detail in the scientific quality, better justification is needed.

Response: We have provided the following description on our WOE assessment approach: "This approach, tailored toward the needs of AOPs, was based on criteria and metrics related to data quality and causality (i.e., the strength of causal linakge between key events). The methodology consists of three main steps: (1) assembling evidence (preparing the AOP), (2) weighting evidence (criteria weighting and scoring), and (3) weighting the body of evidence (aggregating lines of evidence). We adopted the General Assessment Factors (GAF) established by the US EPA as the criteria for data quality evaluation, and a set of nine criteria known as Bradford Hill criteria to measure the strength of causal linkages (see Table below)." If one is interested in learning more details about our approach, we would recommend s/he to take a look at Collier *et al*. 2016.

Regulatory Applicability

I believe that the regulatory applicability of the AOP is well described, and that this AOP could be used by regulators, given sufficient documentation.

Response: We appreciate this comment.

Overall Assessment

This AOP is important, but needs better documentation.

Response: Please see our responses above with regard to better documentation.

Reviewer 2 (R2)

General comments.

- Abbreviations or acronyms should be described when first used in each section of the AOP.
- The AOP has to be checked for grammar, use of present and past tense, typos etc. Some sentences are very long and contain a lot of information and the readability may benefit from splitting such sentences.

Response: We'd appreciate if the reviewer could be more specific and point out what abbreviations, acronyms, grammatical errors, typos and long sentences need us to pay attention because this would save us much time in searching for the errors and correcting them.

Specific comments.

1. It is not exactly clear from the text what exactly comprises the paroxysmal depolarizing shift. For instance, in Event 616 (Occurrence, A paroxysmal depolaizing shift) it is stated that "in epileptic seizures a large group of neurons begin firing in an abnormal, excessive, and synchronized manner, which results in a wave of depolarization known as a paroxysmal depolarizing shift. Normally after an excitatory neuron fires it becomes more resistant to firing for a period of time…. However, in epilepsy the resistance of excitatory

neurons to fire during this period is decreased…. This then results in a specific area from which seizures may develop, known as a "seizure focus".

In the section below it is stated that “epileptiform activitiy consists of a sustained neuronal depolarization resulting in a burst of action potentials, a plateau-like depolarization associated with completion of the action potential burst, and then a rapid repolarization followed by **hyperpolarization.** This sequence is called the paroxysmal depolarizing shift (PDS)”.

So the first description suggests that the sequence leading to and including a sustained depolarization is the PDS, which is then followed by hyperpolarization, while the second description suggests the the hyperpolarization that follows the period of sustained depolarization is also part of the sequence of events in the PDS. This point might be clarified in the AOP.

Response: PDS can refer to both “the wave of depolarization as a result of a large group of neurons firing in an abnormal, excessive and synchronized manner” and “the sequence of depolarization-repolarization-hyperpolarization at the level of single neurons”. The first definition came from Lomen-Hoerth and Messing (2010) and Somjen (2004), whereas the second can be found in Bromfield et al. (2006).

2. In Event 667 (Binding at picrotoxin site, iGABAR chloride channel), figure 1, the structure of the ionotropic GABA receptor is presented. Contrary to what is suggested in the accompanying text, the picrotoxin binding site is not clearly indicated in the figure. From other parts of the AOP it can be read that the picrotoxin binding site is located at the cytoplasmic end of the transmembrane channel. Could the authors confirm that the picrotixin binding site is located at the large intracellular domain between TM3 and TM4? Is it possible to indicate this in the figure?

Response: According to Olsen (PNAS 2006 April, 103 (16) 6081-6082), ScienceDirect (https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/picrotoxin), and Carpenter *et al*. (Chem Res Toxicol. 2013, 26(10):1444-54), residues (e.g., those of TM2) lining the chloride channel at the cytoplasmic (N-terminal) end participate in the binding site for picrotoxin. We have stated this in the legend of Figure 1: “The large extracellular N-terminus is the site for ligand binding as well as the site of action of various drugs”. We also pointed out that “each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein–protein interactions as well as the site for post-translational modifications that modulate receptor activity”. In order to further clarify this, we modified the legend into “The large extracellular ..... various drugs (e.g., picrotoxin and dieldrin)”. Now, we hope that we have clearly indicated the location of picrotoxin binding site.

3. In Event 667 (Binding at picrotoxin site, iGABAR chloride channel), in the section on “overview for molecular initiating event” the description is very concise. The evidence supporting the MIE should be described in more detail.

Response: In this section, we cited five references: Kalueff (2007), Chen *et al*. (2006), Sander *et al*. (2011), Carpenter *et al*. (2013), and Zheng *et al*. (2014). They cover review and research papers as well as experimental and computational evidence supportng this MIE. We believe this

is comprehensive and sufficient. If one wants to know more info or need more detailed descriptions, we would recommend to read the referenced papers and other sections in Event 667.

4. In the KER modules, under quantitative understanding of the linkage the paragraph starts with: ”Is it known how much change in *the first event* is needed to impact the *second*?”. It would improve readability of the KER modules if the first and second event could be specified in this sentence.

Response: We believe this is one of the three general questions posted for us to answer for each KER in the section “Quantitative Understanding of the Linkage”, i.e., “Is it known how much change in *the first event* is needed to impact the *second*?” We think it would be more appropriate for the AOPWiki programmers to modify their script so that the first and second event names can be automatically changed for different KERs.

5. In the description of how Event 682 (Generation, Amplified excitatory postsynaptic potential) works a number of changes observed in epileptogenesis are decribed (last 5 lines of the paragraph). This information is very concise and could be expanded upon.

Response: It was our intention to provide accurate information in a concise style. We would recommend to read Lopantsev *et al*. (2009) if one is interested in knowing more details.

Response to charge questions

Scientific Quality

- Does the AOP incorporate the appropriate scientific literature?

Yes. The induction of epileptic seizures by picrotoxin has been well studied over decades, with many studies addressing this topic. In the current AOP the scientific literature is, although not exhaustively, adequately covered.

Response: Thanks for the remarks.

- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

In general the description of the different key events and key event relationships, alternative theories, datagaps et cetera have been well covered in the AOP. However, as stated above, certain sections would benefit from a more detailed description.

Response: Thanks for the remarks. With regard to the request for more detailed descriptions, we feel that we have provided sufficient information for each section. Even though some of them may seem to be concise and short, this is our style that we wish readers and reviewers can bear with us.

Weight of Evidence

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

The weight of evidence for the different elements of this AOP is generally moderate to strong. The weighing and qualifications of the evidence for the relationship between MIE, key events and adverse outcome are acceptable.

Response: Thanks for the remarks.

Regulatory Applicability

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

This AOP can be used for hazard identification, and as such may be particularly of use for manufacturers of chemicals as an early screening method in the development of new chemicals.

The AOP may provide a mechanism of action for seizures observed in in vivo studies. Based on this AOP chemicals may be grouped into Cumulative Assessment Groups (CAG) and the risk of simultaneous exposure to chemicals belonging to such a CAG may be assessed (Cumulative Risk Assessment). At present this AOP (as is also true for other AOPs) may have limited applicability for regulatory purposes, however, as the methodology for hazard and risk assessment of chemicals are likely to evolve to more in vitro based methods the importance of this AOP for regulatory purpose may increase.

Response: Thanks for the remarks. We agree with this reviewer that a well-defined AOP can enable the development of novel in vitro assays for regulatory application in the near future.

Overall Assessment

- What are your overall conclusions of the assessment of this AOP?

The AOP describes the MIE, key events, key event relationships and adverse outcome for the epileptic seizures that are observed after picrotoxin exposure. The mechanism of action has been extensively studied and is well presented in the current AOP.

Response: Thanks for the remarks.

Reviewer 3 (R3)

Scientific Quality

- Does the AOP incorporate the appropriate scientific literature?

Most of the reported evidences are based on review-style bibliography or on works describing the pathophysiology of epileptic seizures. There is a lack of bibliography more specifically focused on the MIE and first KEs of the stressors. For example:

For the MIE "Binding at picrotoxin site, iGABAR chloride channel", the literature is plenty of data on the inhibition by neurotoxic pesticides of the 35S-TBPS binding to the GABAA receptor (references by Casida, Eldefrawi, Olsen, Squires, Suñol and Rodriguez-Farre,…). Instead, it is written in Page 21: "It has been extensively documented that noncompetitive ion channel blockers such as picrotoxin, lindane, α-endosulfan and fipronil act through binding to iGABARs (Chen et al. 2006)." This sentence is an introductory comment in the paper by Chen et al., 2006, but there is no a demonstration of the antagonism of these compounds on the GABAAR in this paper. Authors should also refer to the original contributions that experimentally defined the non-competitive blocking of the GABAA receptor by these compounds.

Response: If the reviewer may go through the entire article of Chen et al. (2006), all four chemicals (picrotoxin, lindane, α-endofulfa, and fipronil, see Figures 1 and 6 for chemical structures) were investigated through mutagenesis, binding, and molecular modeling experiments. This is one of the few studies that provided direct evidence on the binidng site of non-competitve antagonists (NCAs) in iGABAR. We have come across numerous studies that only performed 35S-TBPS binding experiments, which can't serve as evidence of NCA binding to a specific site in iGABAR. While writing this response, we also checked for new evidence and found Casida and Durkin (2015) (Pesticide Biochem Physiol. 121: 22-30), which has been added to the AOP.

As the reviewer may be aware of, there are decades of research in GABAA/iGABAR antagonism-mediated epileptic seizure. Due to the large body of original research papers, we chose to cite reviews over original research, as the reviewer has noticed. However, the example given above by the reviewer is one of the exceptional cases.

There is a lack of bibliography showing that the stressors induce convulsions in humans or mammals. For example, some references can be added:

- convulsions have been described in humans after non-intentional ingestion of endosulfan and organochlorine pesticides [Moses and Peter, 2010; Parbhu et al., 2009; Durukan et al., 2009; Roberts et al., 2004; Moon et al., 2017];
- 13 organochlorine pesticides that binds to the picrotoxinin site at the GABAAR induce convulsions in mice (Cole and Casida 1986)

Response: We appreciate the reviewer providing more references for the stressors. However, for each stressor, we have already cited references as supporting evidence. We would be happy to cite above additional bibliography if the reviewer could provide some more information (e.g., publication title, journal name, volume, and page numbers) because it is very difficult to find them just using the current information (authors and publication year). The only paper we have found is Cole and Casida (1986), thanks to the many clues provided above and below (in the Weight of Evidence section). However, we

have not yet found a copy of this paper and would very much appreciate if the reviewer could provide an e-copy of it.

Cole LM, Casida JE. Polychlorocycloalkane insecticide-induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. Life Sciences, 1986; 39: 1855–1862.

- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

The AOP reflects current scientific knowledge on the binding of antagonists to the GABAA receptor and on the pathophysiological mechanisms of seizures. However, there are some points I would like to stress:

Title of the AOP.- I suggest substituting "leading to epileptic seizures" to "leading to convulsive seizures". Epileptic seizures include both tonic-clonic and absence seizures; you might have seizures that are not epilepsy (like those induced by drugs, high fever…). [Karatas AD, Aygun D, Baydin A. Characteristics of endosulfan poisoning: a study of 23 cases. Singapore Med J. 2006 Dec;47(12):1030-2 report that after endosulfan poisoning "Seizure types were generalized tonic-clonic in 16 patients (84.2 percent), and focal seizures in three patients (15.8 percent"].

Response: We'd like to begin with differentiating the definitions for epilepsy, seizure and convulsion. According to Sarah Shalev, M.D. Epilepsy Fellow, University of California at San Francisco (https://www.epilepsy.va.gov/Library/VAepilepsybasics.pdf, see page 6), epilepsy is a disorder characterized by recurring seizures (also known as "seizure disorder"); seizure is a brief, temporary disturbance in the electrical activity of the brain. The reviewer is right that not all seizures are epilepsy (i.e.m seizure > epilepsy). But, AOP10 deals specifically with epilepsy (epileptic seizure).

Medical definition of convulsion: An abnormal, involuntary contraction of the muscles most typically seen with certain seizure disorders (see https://www.medicinenet.com/script/main/art.asp?articlekey=88523). **The term convulsion is sometimes used as a synonym for seizure** (see also https://en.wikipedia.org/wiki/Convulsion), but not all seizures are characterized by convulsions. A person having convulsions appears to be shaking rapidly and without control. Other possible causes of convulsions include fever, meningitis, drug or alcohol abuse, poisoning, hypoglycemia, and head injury.

Again, the reviewer is right that convulsions (non-seizure convultions) can be caused by drug, fever, etc. However, again, AOP10 deals specifically with iGABAR antagonism-mediated epilepsy, not seizure in general or convulsive seizure. Hence, **it is inappropriate to expand the scope of AOP10 from epilepsy to convulsion (or convulsive seizure).**

Finally, we'd like to point back to the PDF presentation by Dr. Shalev (available at (https://www.epilepsy.va.gov/Library/VAepilepsybasics.pdf, see page 12), epileptic seizure can be categorized into partial (focal) seizure and generalized seizure (tonic, clonic, absence, atonic, etc.). The publication by Karatas et al (2006) mentioned above by the reviewer reported endosulfan poisoning-induced epileptic seizures (generalized tonic-clonic and focal seizures).

Stressors.- The list of stressors shown in page 2 (picrotoxin, lindane, dieldrin) should be enlarged. As some of the evidences shown in this AOP are based on findings using fipronil and RDX [this compound should be given the complete name when first used], they must be considered as stressors in this AOP. Later in the same page 2, the stressors for the picrotoxin binding sites include fipronil, and dieldrin is not mentioned, whereas it should also be.

Response: We have added a few more chemicals (e.g., RDX, endosulfan, fipronil) to the list of stressors. However, we'd like to acknowledge that it would be a community-wide endeavor to make an exhaustive list of all chemical stresssors sharing this mode of action as described in AOP10.

Key Events organization:

- The order of the KE in the text does not coincide with the order shown in the Graphical Representation (page 21). I rather like the order indicated in the Graphical representation: the reduced GABA-mediated postsynaptic neuronal inhibition (at cellular level) precedes the Paroxysmal depolarizing shift.

Response: We agree with the reviewer that the order of sequence in the KE table does not match the Graphical Representation (Network View). This is beyond our capacity to make any change. We believe that it is either a bug or an error imported from the older version of AOPWiki. Hope the programmer can be alerted to modify the tabulated KE sequence accordingly.

- The KEs "decreased Cl- conductance" and "reduced neuronal synaptic inhibition" could be merged. Since the membrane conductance is determined by the degree of permeability of the cellular membrane to chloride ions it can be measured by the movement of mass (as with the 36Cl- assay) and by the electrical charge (by electrophysiology). Either as merged KEs or as separate ones, bibliography on the last methodology is abundant [but it is not in the text] showing the decrease of chloride conductance induced by PTX and neurotoxic pesticides (for example, works by Narahashi, Akaike, Sattelle, among others).

Response: We respectfully disagree with the reviewer on merging these two KEs. Even though they can be measure by the movement of radio-labelled Cl- or Cl- sensitive fluorescence dye, or by electrophysiological methods, these two KE occur at different organizational levels. Based on our understanding, KE64 (Chloride conductance reduction) occurs at the receptor level, whereas KE669 (Reduction in neuronal synaptic inhibition) occurs at the cellular/tissue level. Also, we did provide reference on the electrophysiological methods in KE669.

Page 11: Occurrence, epileptic seizures. Like for the title, substitute "epileptic seizures" by "convulsive seizures".

Response: See our response above.

Weight of Evidence

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

Yes, but my previous comments also apply

The authors provide weight of evidence for the KE 1 and 2 in relation to some of the stressors. For KE 3 and 4, the weight of evidence is provided for the pathophysiology of epilepsy, but there are no evidences that the stressors specifically produce these KE. Also, they do not provide much evidence for the adverse outcome in relation to the stressors of the AOP

Response: As the reviewer is aware of, there are numerous studies linking stressors to KE3 and KE4. Pathophysiological evidence for epilepsy was used to illustrate the mechanistic studies where researchers tried to figure out the detailed molecular, physiological and cellular mechanisms (instead of a "black-box" type of investigation where one only measure the input and the output).

In Page 22: Strength, consistency, and specificity of association of adverse effect and initiating event.- "Literature evidence strongly and consistently supports such a forward association, i.e., binding to the picrotoxin site leads to epileptic seizures (see reviews Gong et al. 2015; Bromfield et al. 2006; Raymond-Delpech et al. 2005; Treiman 2001; Dichter and Ayala 1987)". In addition to the reviews, the authors might like to cite original works by Cole and Casida, 1986 for 13 neurotoxic pesticides acting at the PTX site ("Convulsions are evident only at doses and times resulting in 30-70% TBPS binding site inhibition "; "62 ± 4% binding site inhibition 30 min after their LD50 doses with 32 ± 3% inhibition at one-half and 6 ± 3% inhibition at one-quarter of their LDs0 doses"), and Squires et al., 1984 who show that Inhibition of [35S]TBPS binding in rat brain correlates with the convulsing dose for convulsing pentylenetetrazole (PTZ) anàlogues.

Response: We would be happy to cite Cole and Casida (1986) and Squires et al. (1984) if the reviewer could provide reprints of these publications because we have not yet had a chance to acquire and read them.

Regulatory Applicability

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

I agree with the comments of the authors in "Considerations for Potential Applications of the AOP". This AOP can be used to establish the mode of neurotoxicological actions for chemicals capable of binding to the picrotoxin convulsant site of iGABARs. It can also be applied to risk assessment where AOP can assist in predictive modeling of chemical toxicity.

Response: We appreciate the remark.

Overall Assessment

- What are your overall conclusions of the assessment of this AOP?

The AOP is well constructed. The involvement of the GABAA receptor on the convulsions induced by antagonists acting at the picrotoxinin binding site is well supported. However, more specific bibliography need to be incorporated to increase the weight of evidence based on what it is known for the specific stressors.

Response: Please see our responses above with regard to bibilography and weight of evidence.

Reviewer 4 (R4)

As for the binding assay, radioligand [35S]TBPS binding assay can be used to determine the binding properties. Is there enough information for the binding activity?

Response: We don't quite comprehend the reviewer's question. What did the reviewer mean by "enough information for binding activity"? Does the reviewer want to know what information can be obtained or derived from the binding assay and whether the obtained info can sufficiently support the conclusion on binding activity? Please clarify.

non-competitive channel blockers (e.g., fipronil, lindane, picrotoxin and alpha-endosulfan) indirectly modulate the iGABAR activity (i.e., alter the response of the receptor to agonist) by noncompetitively binding at or near the central pore of the receptor complex (e.g., the picrotoxin site), an allosteric site distinct from that of the orthosteric agonist binding site, and inducing a conformational change within the receptor (Ernst et al. 2005; Johnston 2005).

I am not sure whether binding sites other than pictoroxin site is important to understand the iGABAR chloride channel.

Are there any information about the sites for non-competitive channel blockers?

Response: There have been quite a lot efforts in characterizing the binding sites (including the picrotoxin site). Such info about binding sites is certainly important for improving the understanding of how iGABAR chloride channel responds to modulators (non-competitive antagonists). Current knowldege is that the picrotoxin site is located at the large extracellular N-terminus of TMs that line up the chloride channel. Many computational and experimental efforts (e.g., Chen et al. (2006), Sander et al. (2011), Carpenter et al. (2013) and Zheng et al. (2014)) have attempted to identify the exact residues (amino acids) that play essential roles in modulating conformational changes within iGABAR and blocking the chloride channel.

Reduction, Ionotropic GABA receptor chloride channel conductance

It is difficult to define the methods of Cl- channel inhibition. I am afraid that there is not enough information for GABA receptor chloride channel conductance.

Response: As explained in the Sectional Guide (viewable if pointing your mouse to the "?" mark to the right of sectional heading), the aim of section "How It Is Measured or Detected" is not to provide detailed protocols, but rather to capture, in a sentence or two per method, the type of measurements that can be used to evaluate the KE and the relative level of scientific confidence in those measurements.

Generation, Amplified excitatory postsynaptic potential (EPSP)

EPSPs are usually recorded using intracellular electrodes. See Miura et al. (1997) and Bromfield et al. (2006) for details.

EPSP amplitudes can be measured by Voltage-Sensitive Dyes.

Response: We have added the method. Very much appreciate the reviewer's suggestion.

Occurrence, Epileptic seizure

Micro-electrode array (MEA) system can be used to detect spikes. I suggest that you can incorporate MEA data to determine neural activities. There are a lot of MEA data using seizure compounds in rat hippocampal neurons.

Considerations for Potential Applications of the AOP (optional)

It is important to understand the scope of application. Which CNS area is to apply the AOP? Is this specific for embryonic neurons? How about adult neurons? The AOP does not distinguish between embryonic and adut.

Response: We have indicated that this AOP is applicable to adult brain. We addressed the distinction between embryonic/immature brain and adult brain in section "Domain of Applicability" (see below):

"This AOP may not be applicable to young animals during their embryonic and early developmental stages when GABA acts as an excitatory neurotransmitter due to increased intracellular Cl^- concentration in inmature or developing neurons (Taketo and Yoshioka 2000; Galanopoulou 2008)".

According to the explanation provided in the Sectional Guide (see earlier response for how to view it), this optional section is for discussion of the potential applicaiton of the AOP to support regulatory decision-making. Hence, we believe the content of our discussion in this section is appropriate.

Scientific Quality

- Does the AOP incorporate the appropriate scientific literature?

The story is well written and the molecular pathway is easy to follow.

Response: We appreciate the remark.

- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

The AOP should describe more techniques to be used. The electrophysiology data are difficult to be reproducible, because electrophysiological experiments require expert technique and experience.

Response: We have added more detection methods as suggestion. As for the reproducibility of electrophysiology data, we are not in the position of evaluating or recommending any specific methods, but rather to provide info about all the methods we are aware of.

Weight of Evidence

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

This AOP is based on the mode of neurotoxicological actions for chemicals capable of binding to the picrotoxin site of iGABARs. The mechanistic approach is reasonable to understand from GABAR to convulsion.

Response: We appreciate the remark.

Regulatory Applicability

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

Chemicals acting on other types of iGABAR sites (e.g., orthosteric or allosteric binding sites) can be distinguished from neurotoxicants that act at picrotoxin sites. In addition, it is not clear whether the AOP can be applied to developing brain. It seems that the scope is understandable, but somewhat limited.

Response: There are two additional AOPs that will be developed to cover other types of iGABAR binding sites (see Gong et al. 2015, where we described three AOPs). We have answered the question about applicability of this AOP to developing brain earlier.

Overall Assessment

- What are your overall conclusions of the assessment of this AOP?

Basically, I agree with the concept of the AOP. The scope should be further discussed.

Response: If the reviewer refers “the scope” to “Considerations for Potential Applications of the AOP”, we believe that we have already addressed the scoping concern earlier.

Reviewer 5 (R5)

Scientific Quality

- Does the AOP correctly incorporate the critical scientific literature and does the scientific content of the AOP reflect the current scientific knowledge on this specific topic?

The current AOP is been developed to explain the mode of action and adverse outcome (AO) when picrotoxin or similar chemicals such as lindane, dieldrin and α-endosulfan binds non-competitively to the so called picrotoxin site of ionotropic $GABA_A$ ($iGABA_A$) receptors ($GABA_AR$) leading to epileptic seizures. Binding to this site of the GABAergic receptor causes the pore to be blocked thereby preventing inward chloride conductance. The authors have focused primarily on the long established common hypothesis that insufficient GABAergic inhibition and the cascading events that follow, leads to epileptic seizures. They have incorporated many of the critical scientific literature that supports this hypothesis including the classical and contemporary understanding of key events and key events relationships leading to AO. Logically, the discussion was focused on scientific evidence, almost exclusively on $GABA_A$ receptors, the ionotropic receptor. They also mentioned $GABA_C$ receptor. However, they made no mention of the $GABA_B$ receptors, a metabotropic receptor, as to suggest that they play no role in epileptic seizures or binding to the picrotoxin site of the $GABA_A$ is independent of and, therefore, not link to activity of $GABA_B$ receptors.

Response: Both $GABA_A$ and $GABA_C$ receptors are ionotropic whereas $GABA_B$ receptors are metaboltropic. As suggested by the AOP title, we limit the scope of this AOP to iGABARs and ligands binding to the picrotoxin site in iGABARs. Therefore, we purposely avoided $GABA_B$ receptors, which does not suggest at all that they play no role in epileptic seizures. Due to the distincitively different properties of $GABA_B$ receptors, it may be necessary to develop another AOP to cover the role of $GABA_B$ receptors in epileptic seizures as well as the interactions between $GABA_A$ receptors and $GABA_B$ receptors.

The authors hypothesis that epilepsy might be caused by defective inhibition of postsynaptic excitatory potential (EPSP) is partially buttress by many lines of pharmacological evidence of anticonvulsant drugs such as barbiturates and benzodiazepines. These drugs exert their anticonvulsive actions at $GABA_AR$s. This hypothesis is also supported by many lines of evidence showing that some GABAergic neurons are susceptible in animal models of epilepsy. Moreover, epileptic seizures are lost in tissue resected surgically from patients with intractable epilepsy. In addition, mutation in the subunits of $GABA_AR$ has been identified as the basis of some forms of genetic epilepsy.

Nonetheless, multiple lines of evidence have also been posited that epilepsy cannot simple be explained solely by a defective $GABA_AR$-mediated inhibition. The strongest support for this alternative hypothesis is based on the observations that GABAergic agonists exacerbate some types of seizures instead of inhibiting them. One of the most notably examples are the drugs/agonist that potentiate GABAergic inhibition increase absence seizures not suppressing them. This has been explained in relation to the actions of GABA at $GABA_B$ receptors on thalamocortical relay cells. GABA agonist is believed to act by potentiating the actions of GABA to hyperpolarize relay cells causing T-type Ca^{2+} current in relay cells to be strongly de-inactivated, leading to more robust bursts of action potentials in relay cells when the hyperpolarization end; these rebound bursts drive the thalamocortical oscillation (Huguenard JR, 1999 and Snead OC, 1995)[1,2]. Therefore, the authors need to increase the specificity of the KEs of picrotoxin to make it extremely clear that this mode of action is limited to certain types of seizures that are independent of $GABA_B$ involvement and this should be supported by scientific evidence. In addition, the

[1] Huguenard JR. 1999. Neuronal circuitry of thalamocortical epilepsy and mechanisms of anti-absence drug action. *Adv Neurol*.:79: 991-9.

[2] Snead OC. 1995. Basic mechanisms of generalized absence seizures. *Ann Neurol.*; 37(2):146-57.

authors fail to consider the role other cations such as intracellular Mg^{2+} and by extension ATP levels and phosphorylation of $GABA_A$ channels plays in ion channel conductance leading to epileptic seizures.

Response: As we mentioned above, this AOP is limited to non-competitive antagonists acting on iGABAR's picrotoxin binding site that lead to epileptic seizure. We are well aware of the complexity of epilepsy and the fact that many causes for epilepsy remain unknown. To the best of our knowledge, we have yet to find any evidence of $GABA_B$ receptor's involvement in this pathway. As for the roles of other cations (e.g., intracellular Mg^{2+}), ATP level (**what does "by extension ATP levels" mean?**), and phosphorylation of $GABA_A$ channels in ion channel conductance, we would appreciate if the reviewer could provide more info and relevant references. We would be happy to address this issue in section "Uncertainties, inconsistencies, and data gaps".

It's interesting that, although the authors described eloquently the pathway by which picrotoxinin binding causes inhibition of $GABA_A$ receptor leading to inhibition of inward chloride conductance, they seems to suggest that, once depolarizations occur downstream, the period of hyperpolarization can then be mediated by either Ca^{2+}-dependent K^+ channels or GABA-activated Cl^- influx. They should explain which one of the mechanisms is operating or perhaps if both mechanisms are operating simultaneously. It is not clear to me, if the authors are suggesting that during block of the GABAergic channel by picrotoxin; somehow it can be reactivated by binding of GABA leading to Cl^- influx. If this is so, then the authors should provide the evidence.

Response: We regret that some text in our AOP caused confusion to the reviewer. We would appreciate if the reviewer could provide specific info as to what sentences in what section in our AOP are confusing. This would get us better oriented.

It was correctly pointed out by the authors that the AOP may not be applicable to young animals due to the excitatory neurotransmitter actions of GABA to increased intracellular Cl^- concentration during development of the nervous system. They explained that, in mature neurons, recurrent and prolonged seizures may trigger a pathological reemergence of immature features of $GABA_A$ receptors, which compromises the efficacy of GABA-mediated inhibition. Furthermore, immature neurons with depolarizing GABAergic signaling, the physiological and pathological regulation of this system is completely different, possibly contributing to the different outcomes of early life seizures. The sponsor should provide additional scientific information or explanation as to the human relevance of knowing this information given that many young kids do suffer from epileptic seizures and if those seizures are not familiar then what MOA explains them.

Response: As we explained earlier, epileptic seizures may have a wide variety of causes or MOAs, some of which remain unknown. We feel that the human relevance of the inapplicability of this AOP to immature/developing neurons is well beyond the scope of this AOP.

Weight of Evidence

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

I agree that the many lines of evidence in different animal species and test systems do strongly support the binding of picrotoxin to the so-called picrotoxin binding site of the iGABAR channel leading to inhibition of inward chloride ion conductance. The authors correctly pointed out that some of the major sources of uncertainties are the wide diversity of subunit genes, in combination with alternative splicing and editing, that leads to an enormous GABAR variety and consequently, variability in functions and sensitivity. They also correctly pointed out that both the weight-of-evidence and quantitative understanding for the generation, amplified excitatory postsynaptic potential (EPSP) leads to occurrence, a paroxysmal depolarizing shift is moderate.

Regulatory Applicability

- What would be the regulatory applicability of this AOP in your opinion?

From a regulatory perspective, this AOP would provide enormous benefit to drug development and by extension speed up regulatory decision making. Having the capability to predict with some levels of certainty, neurotoxicological outcomes of a drug that has like binding properties to picrotoxin could greatly enhance human food safety. Certainly, from the sponsor perspectives it would save enormous time and money getting drugs to the make for treating animal diseases, which is also important from a regulatory standpoint. From a broader perspective, in terms of the AOP ability to predict with high levels of certainty neurological effects of veterinary drug products would be a win-win situation for both the sponsor and regulators but care has to be taken to reduced false positive of any *in vitro* assays that are development.

Drugs that have similar binding properties to picrotoxin could be easily screen using this *in vitro* assay and potentially without the need for the use of many laboratory animals. On the other hand, if we fail to understand many of the nuances with respective to binding of veterinary products to the picrotoxin binding site, we would have failed to protect the public interest and this could be catastrophic. I do believe in the process of trying to transition from animal base decision making to *in silico* method but this cannot be done without regard for proof of concepts, certainly with respect to this AOP where some uncertainty does exist as I have described above. In addition, a drug regulatory we can only accept data that was collected using well validated *in vitro* assays.

Further, as a drug regulator our standard is to assure a reasonable certainty of no harm following consumption of product containing drug residues. Given that this certainty cannot be met in young children based on the limitations of this AOP, its utility for discriminating between drugs that has neurotoxicological effects and specific epileptic potential is greatly diminished without have a better understanding of the MOA in young children.

Response: We agree with the reviewer on the above opinion.

Overall Assessment

1) **What is your overall assessment of the AOP?**

This AOP is a logical and valid attempt by the authors to connect the scientific evidence to some of the long held understanding of the causes of epileptic seizures. This AOP describes the most publicized, well researched and popular hypothesize and naturally is strongly buttressed by many lines of incomplete scientific evidence. The authors have presented a very strong and plausible arguments and many KE to explain and to connect many of our scientific understanding of the causes of epileptic seizures. However, they did not discuss many of the data gaps as they built there AOP model for one of the most complex central nervous system maladies. While I do understand that the AOP is a living document, I believe that a genuine attempt has to be made to either be explicit about the limitations of interpreting the AOP or make the AOP more specific to the kind of epileptic seizure that is best describe by this AOP.

Response: We would appreciate if the reviewer could provide more specifics about the data gaps that the reviewer is concerned.

I think some of the limitations of the scope of this AOP could be missed by users, if we fail to improve the AOP specificity, in terms of what type of epileptic seizure is explained by this AOP model or what seizures are not explained by this AOP and its limitations to adult brain. Its overall regulatory relevance, for animal drug residues that are consumed by humans is extremely limited to adults and we typically regulate animals' drug residues for all stages of brain development. Nonetheless, it could be a useful tool for human drug regulation.

Response: This AOP is applicable to both human and animals because the genetic and functional convervation of iGABARs across the animal kingdom. We have identified several limitations of this AOP (e.g., inapplicability to young developing brain).