

Adverse Outcome Pathway External Review Report

AOP 54: Inhibition of Na⁺/I⁻ symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children

Short title: NIS inhibition and learning and memory impairment

This AOP lists eight key events leading from the molecular initiating event (MIE) inhibition of Na⁺/I⁻ symporter (NIS) to the memory deficit in children (AO). The proposed AOP states that the MIE subsequently 1) decreased thyroid iodide uptake (KE 425) which leads to 2) a decrease in TH synthesis (KE 277) which leads to 3) a decrease in T4 in serum (KE 281) which leads to 4) a decrease in T4 levels in neuronal tissue (KE 280) and 5) BDNF release reduction (KE381), then 6) an altered GABAergic interneurons morphology and function (KE 851) leading to a 7) a decrease in synaptogenesis (KE 385) and 8) decreased neural network function (KE 386) to finally adversely affect learning and memory (AO).

This document is the final report established in May 2018 of an external review started in January 2018. All reviewers mentioned and acknowledged a huge work on this AOP. They agreed on the quality of scientific content with few gaps in scientific literature highlighted in their review. They found the weight of evidence well balanced most of the time but the rationale of KE381 (BDNF) was questioned by a reviewer who found it difficult to understand why this key event so crucial in this AOP and not in really close AOP, sharing a lot of Key events (for example AOP42). Therefore, reviewers did not agree on the direct applicability for regulatory purpose and ask for revisions before further considerations.

Authors did a massive revision before the teleconference (TC) and almost all points raised by the authors were addressed. Overlapping as well as specific issues were raised at the TC. Lasting issues were fixed by the authors. The last version of the AOP was submitted to the authors who validated the revised version of AOP for consideration at the annual OECD meeting. All revisions have been incorporated on the AOP wiki in May 2018.

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1. Synthesis of main issues of the review

Four independent volunteer reviewers were selected among many skilled candidates. Selection was driven by their expertise in thyroid signalling regulations and/or health public concern. Particular attention was taken in balancing reviewers from academy, industry and from different countries around the world.

The AOP 54 describes “Inhibition of Na⁺/I⁻ symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children”. It has been reviewed during January/February 2018 by a team of 4 reviewers (See annex 1)

- Dr Marta Axelstad (DTU_Danemark)
- Dr Ellen Hessel (RIVM_Netherlands)
- Pr Frances Carr (University of Vermont, USA)
- Dr Angela Leung (University California UCLA_USA)

Reviewers were asked to work on the snapshot that was prepared at the beginning of 2018 and to reply to the following questions regarding different aspects of the AOP:

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. *Weight of evidence:*
 - *Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?*
3. *Regulatory applicability:*
 - *Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?*
4. *Conclusion:*
 - *What are your overall conclusions of the assessment of this AOP?*

2. Introduction and background to specific AOP

OECD AOP 54 “ **Inhibition of Na⁺/I⁻ symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children**” with the short title: NIS inhibition and learning and memory impairment can be found at <https://aopwiki.org/aops/54>.

This AOP establishes the link between the inhibition of iodide/sodium symporter inhibition and the consequences on learning and memory deficits in children.

The authors of this AOP are Alexandra Rolaki, Francesca Pistollato, Sharon Munn and Anna Bal-Price* (*corresponding author: anna.price@ec.europa.eu)

AOP 54 describes how inhibition of Na⁺/I⁻ symporter (NIS) could affect memory deficit (AO).

The function of Na⁺/I⁻ symporter (NIS) is critical for the physiological production of TH levels in the serum, as it is a membrane bound glycoprotein that mediates the transport of iodide from the bloodstream into the thyroid cells, and this constitutes the initial step for TH synthesis. NIS is a well-studied target of chemicals, and its inhibition results in decreased hormone synthesis (KE277) and secretion into blood (KE281) leading to subsequent TH insufficiency in the brain (KE280) with indirect detrimental effects in neurocognitive function in children (AO).

More precisely AOP54 describes developmental neurotoxicity (DNT) effects induced by the decreased levels of TH in the blood (KE281) and consequently in the brain, as a result of NIS inhibition. Many environmental chemicals have been reported to disrupt iodide uptake, but the studies that have been focused on NIS inhibition are mainly restricted to perchlorate and some small ionic or drug-like molecules. As thyroid hormones (TH) are essential for brain development, maturation, and function as they regulate the early key developmental processes such as neurogenesis, cell migration, proliferation, myelination and neuronal and glial differentiation. Normal brain development and cognitive function in mammals relies on sufficient production of TH during the perinatal period.

The proposed AOP states that the MIE subsequently 1) decreased thyroid iodide uptake (KE 425) which leads to 2) a decreased in TH synthesis (KE 277) which leads to 3) a decrease in T4 in serum (KE 281) which leads to 4) a decrease in T4 levels in neuronal tissue (KE 280) and 5) BDNF release reduction (KE381), then 6) an altered GABAergic interneurons morphology and function (KE 851) leading to a 7) a decrease in synaptogenesis (KE 385) and 8) decreased neural network function (KE 386) to finally adversely affect learning and memory (AO). The graphical representation of the AOP is accessible on the figure 1

The version modified in January 2018 was submitted for an external review.

This AOP was last updated may 2018.

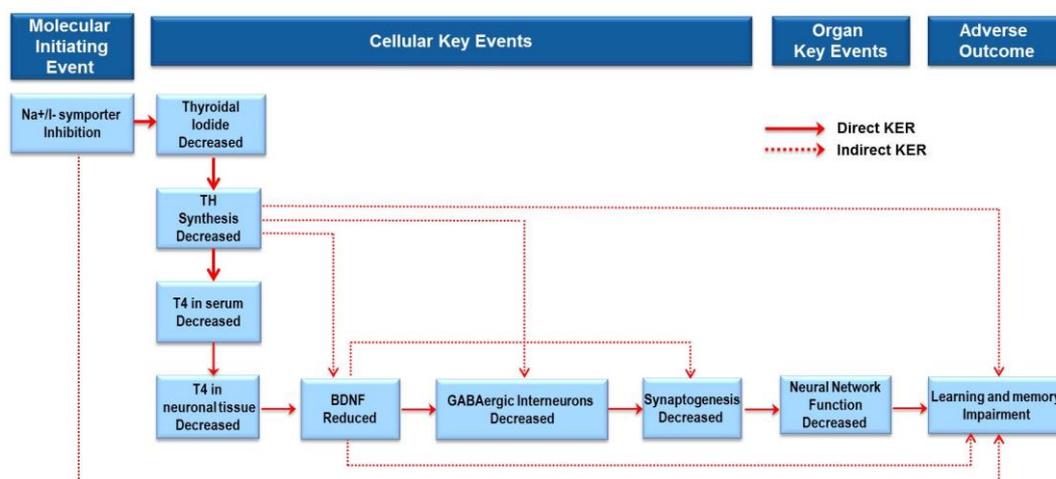


Figure1: Graphical representation of AOP 54 “ Inhibition of Na^+/I^- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children ”

2.1. Scientific quality

The reviewers all agreed on the high scientific quality of the AOP.

They found however that some particular points needed attention and that complementary information should be provided or discussed in order to fit the current conclusions given by the authors.

Authors were asked to revise the AOP in adding some missing conflicting or recent supporting literature.

Does the AOP incorporate the appropriate scientific literature?

All reviewers agreed on the high scientific quality of the AOP. They found however that some particular points needed attention and that complementary information should be provided or discussed in order to fit the conclusions given by the authors. Authors were also asked to revise the AOP in adding some missing conflicting or (recent) supporting literature.

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

Some clarifications and additions were required from the reviewers. The main issues are listed thereafter but extensive reviews were provided by the four reviewers. The complete reviews can be found in Annex 2.

Molecular initiating Events (MIE): Inhibition, Na^+/I^- symporter (NIS)

Iodide intake inhibitors (perchlorate, thiocyanates, nitrates) could target NIS. However, the reviewers raised different issues which were discussed at the teleconference (TC).

- 1) Other stressors than perchlorate, thiocyanates or nitrates are cited as “strong” however dysidenin and aryl-trifluoroborates are not particularly strong but more probably low to moderate (R#3)

- 2) Other parameters could influence the effects of a given chemical stressor. Such parameters as age, gender, pregnancy as well as thyroid status and dietary levels should be described in greater detail in the AOP (R#2 and #3 #4 for thyroid status).
- 3) Recent available data in scientific literature indicate that exposure to perchlorate (among other compounds) during pregnancy is detrimental. R#2 and R#3 suggested that life stage applicability of the **MIE ranged from moderate to strong**. However, Reviewer#1 showed and documented that—evidence for perchlorate leading to cognitive defects needed discussion or additional literature. Indeed, three independent studies not listed by the authors (York et al 2004 & 2005, vanWijk et al 2008, Gilbert & Sui 2008) described an effect of perchlorate on TH levels but were not showing consistent data on adverse behavioral effects in the offspring.

Key Events

KE 281-T4 in serum, decrease:

Two reviewers (R3 and R4), highlighted the need to recommend some standardized methodologies for TH measurements as these are numerous (HPLC, MS, ELISA, RIA) and with different sensitivities. R#4 asked also to mention that blood sampling should be controlled for external factors such as circadian rhythm or food intake.

KE 280 T4 in neuronal tissue, decrease _

As circulating TH levels are not necessarily reflecting brain TH levels, TH levels are different within brain structures. Therefore, the dissection method is crucial for reproducibility and should be clearly mentioned (R#4).

KE 381-Reduced levels of BDNF

All reviewers indicated that BDNF levels reduction is central to the AOP and needs refinement.

- 1) R#1 finds it critical that BDNF reduction is not included in others AOPs sharing same key events and Adverse Outcome. This will need discussion at the TC.
- 2) R#2 questions the dose dependent relationship between T3 levels and BDNF
- 3) R#3 argues that BDNF levels as a cornerstone is mainly driven by accessibility of human data conversely to other consequences of TH decrease. However, plausibility of an hippocampal deficit is stronger than that of BDNF reduction. Focus on hippocampus and/or cortex is also put forward by R#4.

Moreover, R#1 highlights that, in the WoE section, five studies found to be relevant for BDNF by the authors did not report measurements of BDNF mRNA or protein levels. A discussion on why these studies were favoured over others dealing with BDNF is needed.

Key Events relationships

- Regarding the presentation on harmonization and redundancies suppressions are needed (R#1, R#2). For example, a summary of the weight of evidence is provided on p24 whereas a summary/discussion is provided on p26.
- R#1 suggests reorganizing KE and KERs for which *in vitro*, *in vivo* as well as epidemiological data are mixed together.
- Relationship between perchlorate exposure and thyroid effects human needs further discussion (R#1). The reviewer pointed out that all cited studies reveal a non-significant effect of perchlorate and thyroid hormones in humans; a discussion should be added on the basis of confounding effects and how to consider the apparent lack of relationship into the AOP.
- R#3 reminds authors that TH levels can have influence beyond synaptogenesis (neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone projections). R#4 suggests adding an indirect KER as

events other than synaptogenesis can modify BDNF levels. Therefore, comments should be added for these KERs or strength relationships should be balanced.

2.2. Weight of evidence

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

Weight of evidence was most of the time appropriate clear and accurate.

As mentioned earlier, balance for some KERs needed discussion especially concerning the MIE. For example R#1 remarked that for KER 1503 (Inhibition of NIS leads to impairment in learning and memory) the data chosen was not ideal. The AOP authors included two animal studies investigating the effect of perinatal exposure to bisphenol A (BPA) on cognitive function. While BPA may act as a NIS inhibitor *in vitro*, little evidence pointed to this compound as a cause of marked T4 reductions *in vivo*. Reviewers also mentioned the need to harmonize information in KE specific to this AOP with the others in terms of avoiding redundancies. Suggestion to improve MIE life stage applicability was also suggested by 2 reviewers.

2.3. Regulatory applicability:

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

Before the teleconference, divergent opinions arose as to the applicability of the AOP for regulatory purposes. Reviewer # 1 considered that this AOP was not ready to be used for regulatory purposes. One of the major gaps identified being the need for BDNF reductions to be quantified. R#2 and R#4 estimated that due to the well established KE and the fact that IATA take into account certain level of uncertainty, a dynamic update of the AOP would allow for regulatory applications in the near future.

R#3 shared this point of view but also reminded that effects of “iodine supplementation” is not known and strengthens other reviewers’ comments about the importance of thyroid status at the moment of NIS inhibition (MIE).

2.4. Conclusion:

What are your overall conclusions of the assessment of this AOP

All reviewers considered the AOP54 well written and covering most of relevant literature. However, reviewers identified some gaps mainly on missing recent or conflicting literature to be incorporated before further considerations. Authors were asked to consider all numerous comments from reviewers to improve the quality of AO54. Considerations about its broader use would be discussed in the light of a revision and new data incorporated.

3. Summary record of the teleconference

3.1. TC agenda

Agenda for the teleconference May 18th

2pm- 2:45 pm_Specific points on AOP 54

- Presentation of specific comments related to AOP54 (reviewer manager)
- Charge question by charge questions reviewer comments
- Authors reply
- AOB

2:45pm -3:30pm_ Overlapping issues on the two AOPs 54 (NIS) and 42 (TPO)

- Brief overview of both AOP 54 and 42 (Reviewer manager)
- Comments on common key events: TH synthesis decreased, T4 in serum decreased, TH in neuronal tissue decreased
- How addressing AOPs sharing Key event(s) AND adverse outcome(s) with different intermediary key events?

3:30 pm- 4:15 pm_Specific points on AOP 42

- Presentation of specific comments related to AOP42(reviewer manager)
- Charge question by charge questions reviewer main comments
- Authors reply
- AOB

3.2. Main issues and responses during the call

At 2pm participants were

Reviewers: Ellen Hessel (EH), Frances Carr (FC), Angela Leung (AL), Marta Axelstad (MA). At 2.45 Alexius Freyberger joined.

Authors: Anna Price (AP) and Francesca Pistollato (FP) with one connection from JRC.

Authors AOP 42 Kevin Crofton (KC) and Mary Gilbert (MG)

Review Manager: Jean-Baptiste Fini (JBF)

AOP54:

Before the TC, authors submitted an extensive revision of AOP 54 based on individual reviewers' comments (Accessible in Annex3).

All reviewers and reviewer manager thanked authors for the massive work done within a short timing. Therefore the TC was more about discussing remaining issues, namely why choosing to focus on BDNF and if there were still uncovered issues raised by reviewers.

Reviewer manager used powerpoint slides to support specific points addressed by the reviewers but only partially addressed or not addressed in the revision because of authors' questioning (slides are accessible in Annex4).

Using a slide from a presentation performed by Nathalie Delrue in December 2017, JBF reminded that AOPs are living documents and that, in any case, there is an objective of “complete AOP”.

Then a brief overview was given on the AOP54.

General comment on the AOP54 before the revision was that despite the high scientific quality of the AOP acknowledged by the reviewers, some particular points needed attention:

Note that *concerns before the revision* are notified in *italic* and **replies from the authors are in bold**.

On molecular Initiative event (MIE)

- *Other stressors than perchlorate, thiocyanates or nitrates are cited as “strong” however dysidenin and aryl-trifluoroborates are not particularly strong but more probably low to moderate* _ **will be done**
- *Other parameters could influence the effects of a given chemical stressor. Such parameters as age, gender, pregnancy as well as thyroid status and dietary levels should be described in greater detail in the AOP* _ **will be included**
- *Recent available data in scientific literature indicate that exposure to perchlorate (among other compounds) during pregnancy is detrimental*_ **Authors provided in the revised version sent before the TC appropriate revision and included the recent literature covering this point.**
- *Major discussion on evidence about perchlorate leading to cognitive defects needs discussion.* _ **new data already incorporated in the revision**
- *Change life stage applicability from moderate to strong*_ **done**

Questions on the KE 381 and KER 444:

Before revision reviewers indicated that BDNF levels reduction needed refinement.

- *One key question was the quantification of dose dependent relationship between T3 levels and BDNF*_ **this specific point was addressed by the authors and new data has been provided.**
- *Two reviewers put forward that BDNF levels were mainly driven by accessibility of human data in contrast to other consequences of TH decrease. Plausibility of an hippocampal deficit being stronger than that of BDNF reduction, shall a focus on hippocampus and/or cortex be preferable?* _ **this point generated an intense discussion. Main argue from the authors (AOP 54 but also 42 present) were that AOP are unique and if one wants to link BDNF with hippocampus gene expression, therefore another AOP should be constructed. Both, AOP54 authors and AOP42 mentioned that the AOP networks linking the two AOP will be a future step in the AOP process. Giving these elements all attendees decided to maintain the AOP in their present form.**
- *Given the revisions provided should it be branched?*_ **this point of discussion was postpone to the second part of TC in overlapping issues.**
- *T4 decrease in neuronal tissue and BDNF reduction is “weak”. Giving the new literature presented by authors in the revision, should it be integrated within the KE “Altered gene expression?” or should it go from weak to moderate or stand as it is?*_ **this specific point was discussed and everybody agreed on the second option changing weak into moderate.**
- *Missing literature in discussion limitations of some effects*_ **All reviewers agreed on the fact that this issue was solved with the revision provided.**
- *TH levels can have influence beyond synaptogenesis (neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone*

projections). Suggestion made on adding an indirect KER as events other than synaptogenesis can modify BDNF levels. **In the paper by Robichaux et al., 2014 different pathways that regulate synaptogenesis are described; however, in this paper it is not discussed which pathways involved in synaptogenesis are under the control of TH signalling. This new KER was not further considered as discussed in the revised version of AOP.**

- *Regarding the presentation on harmonization and redundancies suppressions are needed_Authors said they will remove when unnecessary (Note that KE (and KER) should be seen as independent documents)*
- Non addressed issues because of queries from authors p8, p11, p27,p33, p34 or the revision._ **This point was postpone after the TC, to be addressed by email exchanges.**

Finally, the regulatory aspect was discussed. JBF reminded that AOP are modular, living documents and that AOP networks mentioned by the authors will be the following step.

Before the revision sent on May 16th divergent opinions arose as to the applicability of the AOP for regulatory purposes. One reviewer considered that this AOP was not ready to be used for regulatory purposes. One of the major gaps being the need for BDNF quantifications.

Two reviewers estimated that due to the well-established KE a dynamic update of the AOP would allow for regulatory applications in the near future. The last reviewer shared this point of view and reminds that effects of “iodine supplementation” is not known and strengthens other reviewers’ comments about the importance of thyroid status at the moment of NIS inhibition (MIE).

Given recent literature addition supporting BDNF quantification and massive revision, addressing and discussing all of the points raised by the reviewers, all reviewers agreed on modifying the conclusions of AOP54 to be considered in a short timing for regulatory purposes.

Second part: Overlapping issues.

When comparing AOP 54 with 42, one could see that right after the two different molecular initiating events, three KE are common: KE 277 TH synthesis, decreased; KE 281 T4 in serum, decreased ; KE 280 T4 in neuronal tissue, decreased (see Figure 2). After KE 280 there are two divergent key events which are KE 756 hippocampal gene expression, altered; and KE 381 reduced levels of BDNF.

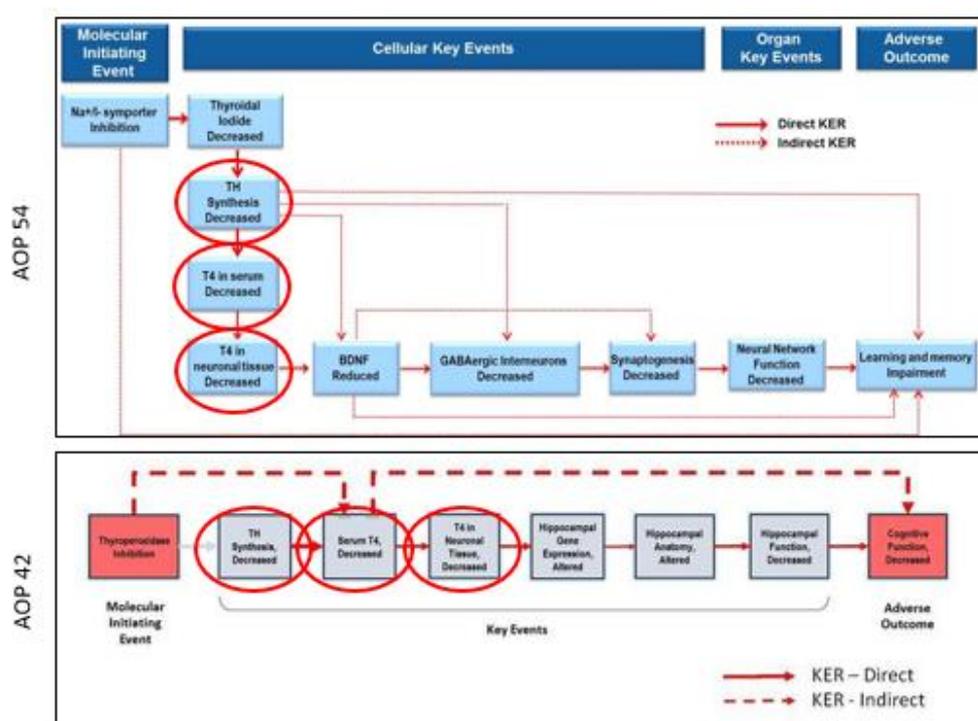


Figure 2: Comparison of the two graphical abstracts if AOP42 and 54. Red circles show common KEs between the two AOPs. Note that the AOP42 graphical abstract is truncated and is not the final version (missing indirect KERs)

On KE 281 T4 in serum, decreased.

On that specific point reviewers highlighted the need to recommend some methodologies for TH measurements as there are numerous (HPLC, MS, ELISA, RIA) with different sensitivities.

KC, MG and AP agreed on the fact that AOP authors are not supposed to suggest, promote technologies and that it is not what they need to do in KE description. They agreed that the different technologies should be listed but not detailed. Giving these elements, reviewers validated the current presentation of KE281.

A related point raised by two reviewers was a “need for standardized methodologies. Due to the argues from the authors of both AOP54 and 42 explaining that authors are not intended to recommend techniques, everybody considered this point cleared.

On KE 280: T4 in neuronal tissue, decreased

One reviewer mentioned that TH levels being different within brain structures, dissection method is crucial for reproducibility and should be clearly mentioned. However giving the discussion which took place at the beginning of the TC on what authors have to provide and not provide, **we all agreed that this was not the role of authors to promote a technique for either TH level measurements (KE 281) or dissection (KE 280). However, everybody agreed on the relevance of the issue raised and asked for a discussion in the revised AOP.**

One major issue was also the “harmonization between AOPs?”

The divergence after T4 levels in neuronal tissue leading to KE 756 hippocampal gene expression, altered; and KE 381 reduced levels of BDNF.

JBF suggested that when two AOPs have different KEs which lead to similar late KEs or AO a branching could be considered.

Another suggestion was to consider KE 381 as part of KE756 if considering only hippocampus. These considerations were discussed but as already stated AOP 42 and 54 are considered different and independent. **Everybody agreed that these pathways could stand separate.**

Last point of harmonization was on the tables showing divergent weight of evidence and quantitative understanding of AOP KERs. For the common KERs, different evidence weights are given. In AOP54 “Thyroid hormone synthesis decreased” leading to “T4 in serum decreased”, evidence and quantitative understanding were respectively strong and strong whereas in AOP42 evidence and quantitative understanding were strong and moderate.

Regarding the KER “thyroid in serum decreased” leading to “decreased T4 levels in neuronal tissue” evidence and quantitative understanding for AOP 54 were strong and weak but moderate and weak in AOP42. **AOP 54 authors agreed to adapt AOP54 in order to fit with AOP42.**

JBF asked AP if she wanted to add something, ask question. She and FP deeply thanked the reviewers for their very relevant comments and the work done on the AOP which made the AOP improved a lot.

The AOP 54 TC ended at that moment and AOP54 authors stayed connected until the 5.25pm when the AOP42 ended.

3.3. Action list

Authors already did before the TC an extensive work in revising the AOP taking into account most of reviewers’ comments.

Actions listed after the TC was therefore quite succinct:

- 1) Obtain the precisions from the reviewers about the following points:
 - *R#1 said. “On page 52, in the WoE section, under the study summaries, there is a short discussion of the relevance of BDNF, and the authors refer to five studies where no effect on BDNF mRNA or protein levels are seen”* Authors could not find the articles referred by the authors.
 - *R#2 There are multiple other stressors which can inhibit NIS that have greater applicability, thus these two are not those which one usually associates with this MIE.* Authors could not find other stressors.
 - *R#4 The discussion of Uncertainties or Inconsistencies (p. 25 27) may be strengthened with new studies that indicate a dose relationship between perchlorate and thiocyanate exposure and thyroxine levels and outcomes.* Authors needed to know which studies reviewer referred to.
- 2) to modify status of KE 381 from weak to moderate.
- 3) to harmonize AOP54 KE and KER scoring with AOP42 according to what was discussed at the TC
- 4) AP to incorporate the modifications on AOP wiki

4. Summary of planned revisions

Numerous planned revisions were listed in the point by point rebuttal sent from AOP54 authors to all reviewers and review manager before the TC (accessible in annex3).

All authors agreed on the planned modifications listed in the rebuttal. Main modifications included additional literature and discussion of uncertainties and inconsistencies for few KEs.

Additional minor points discussed at the TC were:

- Change life stage applicability for MIE from moderate to strong
- From the AOP presentation, harmonization and redundancies suppressions were discusses and planned to be done by the end of May.
- The authors, in the rebuttal, questioned some unclear points. Due to the tight timing at the TC, we all decided to discuss three specific questions by email before the end of May.

First point was the multiple stressors which could be considered at the MIE (R#2). Another question was about other neurodevelopmental defects well-known to result from decreased thyroid hormone levels to be incorporated in KE. Finally R#2 considered that as the AOP has evolved and following the conversation in the teleconference, due authors' excellent job, no further modifications are necessary.

The other two remaining points were discussion of articles (initially not mentioned) required by R#1 and R#3 but without references provided. Therefore reviewers were asked to provide the references and authors agreed and incorporated these last changes in the AOP.

May 30th, authors answered to all questions. Authors did make the modifications and incorporated them in the AOP Wiki in the first week of June 2018.

5. Further discussion

From the review manager's point of view, expectations in terms of reviewing, communication, organisation and reporting are clearer than at the beginning of review process. Review manager suggests to have a common safe space on the cloud on which all documents, reviews and letters would be accessible. And as it is done during manuscript peer reviewing, he also suggests a status indicator in order to get an overview of the external review process.

6. Outcome of the external review

The reviewer panel agreed on the high quality of the work done. Giving the extensive revision provided by the authors, reviewers find that additions and revisions are clear and satisfactory. They also find that the revised AOP ready for final OECD approval and for regulatory applications in a near future.

Annex 1: Table with reviewers' name

AOP title	Links (wiki / snapshot)	Review manager	Reviewers
AOP 54: Inhibition of Na ⁺ /I ⁻ -symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	https://aopwiki.org/aops/54 Snapshot: [HTML] ; [PDF] Internal review AOP 54	Dr Jean-Baptiste Fini (CNRS_France)	Dr Angela Leung (University UCLA_ USA) Dr Ellen Hessel (RIVM_Netherlands) Dr Marta Axelstad (DTU_Danemark) Dr Alexius Freyberger (Bayer_Germany) Pr Frances Carr (University of Vermont_USA)

Annex 2: Individual reviewers' comments

Reviewer #1

Scientific quality:

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

In general much relevant literature has been incorporated into the descriptions of the shown KE and KERs. I do however find that a few issues would benefit from some revision.

1) In the last sentence of the abstract it is stated that "Perchlorate, which is the most potent inhibitor of NIS, has been associated with reduced TH production and **also with cognitive deficits in animals...**". I however do not find that there is very good data to support this argument. A literature search in PubMed (February 2018), revealed that neurobehavioural effects after perinatal perchlorate exposure have been investigated in the following three *in vivo* studies (York et al 2004 & 2005, vanWijk et al 2008, Gilbert & Sui)¹. In my opinion none of the obtained results support this conclusion very well.

York et al. (2004) conclude that: "There were no behavioral effects in the offspring exposed as high as 10.0 mg/kg-day as evaluated by passive avoidance, swimming water maze, motor activity, and auditory startle." In their re-evaluation of the data (York et al. 2005) the authors again conclude that: "The behavioral testing suggests prenatal exposure to ammonium perchlorate does not affect the development of gross motor movements in the pups", and Gilbert & Sui (2008) write in their abstract that: "Perchlorate did not impair motor activity, spatial learning, or fear conditioning".

vanWijk et al (2008) was the only publication to show small effects on neurobehavioural development in perinatally perchlorate treated animals. This paper showed delayed neuro-motor skill during early postnatal development (measured as grip-test and balance beam). This effect was however most probably caused by low postnatal body weight, as the neuro-motor skills were no longer observed later in life, when the animals were no longer low in body weight. The authors found no effect on locomotor activity (open field) in either sex, but did find small but significant adverse effects in learning and memory test (Morris maze) in female offspring (but not in males). However the study was performed with only one dose group exposed to perchlorate only during development, and the behavioural assessments were performed using a group size of 5-8, making the reliability of this study, quite limited.

¹ Gilbert ME, Sui L. [Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat](#). Environ Health Perspect. 2008 Jun;116(6):752-60

van Wijk N, Rijntjes E, van de Heijning BJ. [Perinatal and chronic hypothyroidism impair behavioural development in male and female rats](#). Exp Physiol. 2008 Nov;93(11):1199-209

York RG, Barnett J, Girard MF, Mattie DR, Bekkedal MV, Garman RH, Strawson JS. Refining the effects observed in a developmental neurobehavioral study of ammonium perchlorate administered orally in drinking water to rats. II. Behavioral and neurodevelopment effects. Int J Toxicol. 2005 Nov-Dec;24(6):451-67

York RG, Barnett J Jr, Brown WR, Garman RH, Mattie DR, Dodd D. A rat neurodevelopmental evaluation of offspring, including evaluation of adult and neonatal thyroid, from mothers treated with ammonium perchlorate in drinking water. Int J Toxicol. 2004 May-Jun;23(3):191-214.

In some of these studies, other neurological endpoints than behavior, were adversely affected. There was an apparent increase in the thickness of the corpus callosum in day 12 pups (York et al 2005) and Gilbert & Sui (2008) found dose-dependent deficits in hippocampal synaptic function.

Together, these findings indicate that brain development may indeed be affected by perinatal perchlorate exposure, but all in all, there are no consistent data showing adverse behavioral effects in the offspring (at doses causing altered thyroid hormone levels during development). A discussion of this point is in my opinion detrimental, and is presently completely missing from this AOP. It would be especially beneficial in the WoE discussion of KER (Inhibition of NIS leads to impairment in learning and memory) on page 47. Unfortunately here, only data from the van Wijk et al (2008) study are included and none of the limitations of this study are mentioned. This issue in my opinion needs some revision.

2) In general for many of the KE and KER descriptions, references and result description from in vitro, in vivo and epidemiology studies are mixed together, so that it is hard to discern which data is from which types of studies. A clearer division of the text into results obtained from the different types of studies would ease the reading of the AOP and improve the understanding if it. This is for instance the case on P. 26 (last line), where it is not stated which test system(s) the conclusion is based on - but this is also the case several other places throughout the AOP, for instance in the overall assessment of the AOP (page 61).

3) I find that throughout the document there is too much repetition within some of the KE and KER description. Especially the short study descriptions are often provided both in the “weight of evidence” sections, in the “empirical support for linkage” section and in some cases again under “quantitative understanding of the linkage” (e.g. on p. 24, 27, 40, 44, 53 and other pages). In the AOP 42 this is not an issue, and I find that it would improve the present AOP considerably if this issue was revised throughout the AOP.

4) The discussion of human data in relation to the link between perchlorate exposure and thyroid effects is in my view not fully discussed. First mentioning of this is on page 24, mid page, last sentence - before the “Biological Plausibility” paragraph. Here a reference is made to only one epidemiology study (Braverman et al 2006), not showing any significant link, between perchlorate exposure and TH levels. The paragraph ends with a sentence that the study is “obviously statistically underpowered”, indicating that its conclusions are not very reliable. I would suggest to include number of study participants in order to substantiate this claim, as no study description is included in this KE description under “empirical support for linkage”. Other study references investigating this issue could be provided here.

The discussion of this issue continues on page 27 (line 4-10) because here several other epidemiology studies on this issue are cited. However none of these have actually shown any significant relationship between perchlorate exposure and thyroid effects in humans (Tarone et al 2010, Tellez et al 2005, Pearce et al 2010, Steinmaus 2016b). So in reality all cited epidemiology studies come to the same conclusion as the Braverman et al 2006 study... A more clear WoE analysis, taking all available knowledge into account would be beneficial and a discussion of whether any human data actually show effects on TH status related to perchlorate exposure, would also be helpful to include here.

Weight of evidence:

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

In general yes, with some exceptions, as mentioned above and below.

As also pointed out in my review of AOP 42, if indeed reduced levels of BDNF is a KE leading to impaired brain development, this should be reflected equally in both AOPs (i.e. a revision of AOP 42 is needed). On page 52, in the WoE section, under the study summaries, there is a short discussion of the relevance of BDNF, and the authors refer to five studies where no effect on BDNF mRNA or protein levels are seen. The AOP would however benefit greatly from a short discussion of whether the authors find that these studies are maybe less convincing or more poorly performed than those showing BDNF effects - in order to convince readers why BDNF levels should be a cornerstone in the AOP, relating low TH levels to altered brain development.

The discussion of this issue continues on page 59 (line 6 from the bottom) where the authors conclude that there are no inconsistencies in this KER. I find it difficult to assess whether this is correct and I would suggest to include a bit of discussion on how studies with KO mice or studies with compounds specifically inhibiting BDNF (but with no other effects) could be useful, in order to show the direct causal link.

On page 47, in the WoE discussion of KER 1503 (Inhibition of NIS leads to impairment in learning and memory) the data chosen to support the KER are in my opinion not very convincing. The AOP authors include two animal studies investigating the effect of perinatal BPA exposure on cognitive function (Wang et al 2016 & Jang et al 2012). And while BPA may act as a NIS inhibitor in vitro, little evidence points to this compound causing marked T4 reductions in vivo. To me, it therefore seems much more plausible that the effects of BPA on cognitive function (shown in these two and a large number of other in vivo studies) was probably not NIS (T4) mediated, but possibly caused by the estrogenic or other effects of BPA.

Then some epidemiology studies that are cited, relating exposure levels of perchlorate and PBDEs in pregnant women, to cognitive deficits in their children (Taylor et al 2014; Chen et al 2014; Roze et al 2009). But does the Taylor et al 2014 study actually investigate whether the perchlorate exposed mothers were hypothyroxinemic? If this is not shown, the observed IQ reductions could be related to concomitant co-exposures to other chemicals in these women. And in the Chen et al (2014), and Roze et al (2009) studies, exposure to PBDE could be affecting the developing nervous system through other MoA than via hypothyroidisms, for instance a direct neurotoxic MoA. Based on these studies (or at least the provided summaries) we are not able to tell whether any of the observed adverse effects on neurodevelopment are indeed caused by hypothyroidism (e.g. are due to NIS inhibition).

Regulatory applicability:

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

The key question is in my opinion the quantitative link between BDNF expression and neurological impairment. On page 3, line 13, it is stated that dose-response relationships between TH levels and reduced BDNF expression in the developing brain cannot not be evaluated, as all studies have been conducted in conditions of severe maternal hypothyroidism. I however find it hard to believe that in all performed studies (using different doses of chemicals or iodine deprivation) all T4 reductions were identical, and that it would not be possible to perform some sort of no dose-reponse relationship between T4 and BDNF.

Solving this issue and of course a concomitant inclusion of BDNF measurement into more regulatory studies, would help solve this gap.

All in all, this AOP is in my opinion not yet ready for regulatory use, but could maybe be combined with AOP 42, and hereafter be used for prioritization of chemicals for further in vivo testing.

Conclusion:

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

This AOP has gathered and described much relevant literature, but in my opinion there are still some major revisions needed (as stated above) prior to its proper use.

Reviewer #2**SCIENTIFIC QUALITY:**

Overall AOP incorporates relevant scientific literature to support the relationship between the MIE, inhibition of NIS, reduction of serum thyroid hormones, and learning and memory impairment. The evidence provided supports the biological plausibility of the pathway and associated classification of the stressors.

Observations to be considered:

MOLECULAR INITIATING EVENT

1. Age, gender, pregnancy, as well as thyroidal status and dietary iodine levels affect the impact of perchlorate stressor, inhibition of NIS and serum thyroxine levels. These factors do not seem to be prominent in the AOP. Recent studies provide additional evidence of the importance of age at exposure to stressors.
 - The overview for the MIE, p.2, notes the potential contradictory results from studies that showed a correlation versus no correlation between thyroid parameters and perchlorate in humans.
 - Horton et al. 2015 demonstrated that co-occurrence of perchlorate, nitrate, and thiocyanate alters thyroid function in pregnant women. The contradictory findings may be a result of the confounding mixtures in the environment masking the primary effect of perchlorate.
 - PMID: PMC4641782
 - Taylor et al. 2014 found that higher maternal urinary perchlorate levels correlated with lower I.Q. in offspring independent of thyroxine therapy. These data suggest potentially direct impact on the development of the fetal thyroid gland.
 - PMID: 25057878

These studies represent a body of evidence that perhaps indicates that fetal and neonatal periods of thyroid gland development are critically sensitive to NIS inhibition.

- McMullen et al. 2017 that adolescents, both male and female, are more sensitive to exposure than are adults. This study clarifies that correlation between perchlorate, thiocyanate and serum T4 levels and notes again the absence of significant change in TSH.
 - PMID: 28430972
2. Is the Evidence considered to be only Moderate for Life Stage Applicability (p.2)? Recent studies together with evidence cited throughout the key events suggest Strong Evidence particularly for Pregnancy and Birth to <1 month.

KEY EVENTS

The classification of the evidence in support of upstream event(s) leading (directly/indirectly) to downstream event(s) is justified adequately. AOP referencing the relationship of NIS inhibition to subsequent adverse neurodevelopmental outcomes in mammals and NIS inhibition to learning and memory impairment is strong.

KEY EVENT RELATIONSHIPS

It is helpful to have the same format for each of the relationship sections. For example, a summary of the Weight of Evidence is provided on p.24 and then citations whereas a summary/discussion is provided after citations on p. 26, 27.

3. The statement that “The thyroid system is complex....” P. 24 cites only one study that shows no relationship of perchlorate and thyroid function. It would be more effective to cite a contrasting study e.g. McMullen et al 2017. The sentence is repeated on p.25 under uncertainties. It is more appropriate in uncertainties or inconsistencies.
4. The discussion of Uncertainties or Inconsistencies (p.25 27) may be strengthened with new studies that indicate a dose relationship between perchlorate and thiocyanate exposure and thyroxine levels and outcomes.
5. The KER of decreased T4 in serum and decreased T4 in neuronal tissue is plausible. Given the compensatory mechanisms to maintain adequate and not excessive T4/T3 in brain tissue, the degree to which decreased serum T4 directly corresponds to quantifiable decreased T4 in neuronal tissue is not yet clear. Should this be more directly stated? Nevertheless, that decreased serum TH results in lower brain TH concentrations is well established.
6. Evidence for dose –dependent relationship between T3 and BDNF may be strengthened.

Siu et al. (2010) demonstrated that administration of T3 increases BDNF in rat hippocampus in vivo in a dose-dependent manner.
PMID: 20018181

Mokhtari et al. (2017) established a link between T3 and upregulation of BDNF and learning and memory in an ischemic stroke model in rats.
PMID: 28202057

Weight of Evidence

MIE Evidence supports the classifications. Given more recent studies, the evidence in support of lifespan applicability is growing. The potential for indicating the evidence is strong rather than moderate should be considered (page 2). The scoring within the AOP for KEs, KERs is consonant with the evidence cited. Additional references such as provided above can strengthen the evidence however; the critical citations have been included already.

Regulatory Applicability

Since the AOP covers endpoints that are measured using widely accepted methods, including TH levels and memory, learning and IQ, it is highly probable that it will have broad regulatory applicability. This AOP can provide the basis for standardizing evaluation of classes of chemicals and their biological impact. The weight of evidence and classifications of the KEs and KERs provides an important framework to guide policy/regulatory development.

Conclusion

The AOP is very well developed. The revisions suggested provide possible enhancements to the AOP but the central tenets are strong and well supported.

Reviewer #3

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?**

The broad hypothesis of AOP 54 is based on the strong scientific evidence that thyroid hormone is critical for normal neurodevelopment, such that the MIE of sodium iodide symporter (NIS) inhibition results in several KEs corresponding to decreased thyroid hormone availability and synaptogenesis defects to result in the AO of decreased learning and memory. This AOP is well-written and presented with good biological plausibility.

However, the specified KEs represent one potential pathway toward the AO. Brain development is complex, and there are likely other KEs and AOs which can follow the MIE.

- a. KEs related to decreased neuronal thyroid hormone levels: Thyroid hormone-dependent actions in the brain not only include synaptogenesis, but also neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone projections. The preponderance of these other KEs would be based on primarily animal data, with the exception of hippocampal defects that does have both animal and human data. It would be worth considering to include the hippocampal work of Drs. Mary Gilbert, Joanne Rovet, and colleagues as KEs. Notably, the strength of evidence underlying the relationship between low thyroid hormone levels and hippocampal deficits is likely stronger than that between low thyroid hormone levels and decreased BDNF (regarded as low strength of evidence by the AOP authors and as written about on page 54: BDNF is thought to underlie the effects of developmental hypothyroidism but this notion is based mainly on their common physiological role during brain development rather than on solid experimental evidence [Gilbert and Lasley, 2013].)
- b. AOs: In addition to deficits in learning and memory, decreased IQ in children has specifically been studied quite extensively as a result of maternal hypothyroidism during pregnancy. Both the current AO and decreased IQ are more commonly grouped under the global term of decreased cognition, and thus this could also be added as a critical AO.

2. 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 dysidenin and aryltrifluoroborates as stressors to the MIE are not particularly strong, and probably low to moderate at best. There are multiple other stressors which can inhibit NIS that have greater applicability, thus these two are not those which one usually associates with this MIE.

Regarding the life stage applicability of the MIE specifically, there are significant data supporting the concept that decreased thyroid hormone resulting from NIS inhibition is most relevant during early life when neurodevelopment begins, thus I would recommend increasing the strength of evidence during the three listed life stages from moderate to strong.

Otherwise, I agree with the weight of evidence designations assigned to each of the KEs, KERs, and the overall AOP.

3. Regulatory applicability

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

The point presented in the Considerations for Potential Applications of this AOP would be supported by the evidence presented. As Integrated Approaches and Testing Assessment (IATA) strategies take into account an acceptable level of uncertainty and not all of the intermediate KEs need to be quantified, this AOP would provide an initial basis for the identification of substances to identify chemicals acting through this pathway.

One important consideration that is not noted, however, is that the potential severity of the AO can be mitigated by adequate iodine nutrition to overcome the effects of the MIE; the mention of iodine status is absent in this section and is crucial toward potential regulatory applicability of this AOP.

4. Conclusion

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

Broadly, AOP 54 is well-prepared and has been comprehensively organized. There are some concerns regarding the strength of evidence underlying the KEs and KERs, and there are certainly others which may offer a higher level of evidence linking the MIE with the AO. In addition, the AO is fairly specific, and other neurodevelopment defects well-known to result from decreased thyroid hormone levels are not represented. Finally, regulatory applicability of the AOP must take into account iodine status and supplementation, and further emphasis on this important mediator can be made in the AOP. Overall, the authors have developed a thoughtful summary of the current concepts underlying this pathway.

Reviewer#4**General comments**

The AOP is very well described and of good quality and the available studies are adequately judged.

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?
 - a) For MIE, Inhibition of Na⁺/I⁻ symporter (NIS): please refer to the OECD new scoping document and the references used within this document in the paragraph “how to measured or detected”. Title document: “*New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling*” the NIS assay at page 36-38.
 - b) KE ‘T4 in serum, decreased’ (page 8-11): paragraph “how it is measured or detected”
 - a) Please specify what the advantages and disadvantages are for measuring free and total T4 and T3 and what the preference has to measure this KE.
 - b) Different techniques are mentioned to measure thyroid blood levels. Two are missing namely HPLC-MS and Immuno Luminescence. All the available assays have different sensitivities. Therefore, results including reproducibility and repeatability really depends on the protocol used. Standardization of analysis for this KE is crucial to make comparisons between independent experiments possible and to better judge the effects in TH levels (Chang et al., 2007).
 - c) Please mention that blood sampling should be controlled for experimental factors (such as circadian rhythm or food intake) that might influence the measured concentration measured and the variability of the hormone determination (Döhler et al., 1979).
 - c) For KE “T4 in neuronal tissue, decreased”, paragraph ‘how it is measured and detected’ (page 11-13). Based on the brain region specific levels it is important not to measure whole brain levels but also brain region specific TH levels although that might be quite hard (Constantinou et al., 2005). Please mention that the way of dissecting the brain regions is crucial to draw the right conclusions. It is also possible to measure other aspects of the thyroid functioning since reporters and enzymes affect levels in specific brain regions (Moog et al., 2017).
 - d) For KE “Reduced levels of BDNF”, please focus on a brain region or mentioned the “most likely affected brain regions” such as the hippocampus and cortex and include references (Koromilas et al., 2010, Shafiee et al., 2016).
 - e) Noting is mentioned in the KEs about of the effects of maternal hypothyroidism. It is likely that during prenatal brain development effects of disruption of the thyroid levels might be more severe than during adulthood, although effects of hypothyroidism on neuronal functioning occurs throughout life (Moog et al., 2017, Préau et al., 2015)
 - f) From a neuroscientific point of view, the models measuring the KE are very generally written at this stage. In future neuroscientific expertise on how the KE can be measured properly might help to make the best choice for a model to study the Key event.
 - g) Include references at page 20 by the sentence: “patch clamping technique can also be used to measure neuronal network activity” (e.g. Bosca et al., 2014).
 - h) Thyroid levels can also affect synaptogenesis via other pathways (not only BDNF). The authors can consider including an indirect KER between T4 serum levels and synaptogenesis (Robichaux et al., 2014)?

2. 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 judgement by the AOP developers for the KER is very clearly and accurately described, the available studies are very well judged and all the uncertainties are described accurately.

3. 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 developmental (neuro) toxicity and for identification of endocrine disruptors (thyroid disruptors). Additionally, this AOP can be used and help to unravel the mechanisms of thyroid hormone disruption and the occurrence of learning and memory impairment. Therefore, it is probable that it will be applicable for mechanistic tests as part of an IATA. The AOP is very interesting since it describes an important aspect of thyroid disruption for which many epidemiological evidence is available.

4. Conclusion:

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

I would recommend this AOP for submission. An AOP is intended to be a constantly developing document, the adverse outcome is very important and proven to occur after hypothyroidism. It nicely links epidemiological evidence with mechanistic data. A more specific description of available models to measure the KE is needed in future. Specific expertise on the models from neuroscientist would be helpful, since these KE are difficult to measure. The AOP is very well described and of good quality.

References:

- Bosca, A., M. Martina, and C. Py, Planar patch clamp for neuronal networks--considerations and future perspectives. *Methods Mol Biol*, 2014. 1183: p. 93-113.
- Chang SC1, Thibodeaux JR, Eastvold ML, Ehresman DJ, Bjork JA, Froehlich JW, Lau CS, Singh RJ, Wallace KB, Butenhoff JL. Negative bias from analog methods used in the analysis of free thyroxine in rat serum containing perfluorooctanesulfonate (PFOS). *Toxicology*. 2007 May 5;234(1-2):21-33.
- Constantinou C, Margarity M, Valcana T. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. *Molecular and Cellular Biochemistry*. October 2005, Volume 278, Issue 1–2, pp 93–100
- Döhler et al. (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. *Pharmac Ther* 5: 305-318.
- Koromilas C1, Liapi C, Schulpis KH, Kalafatakis K, Zarros A, Tsakiris S. Structural and functional alterations in the hippocampus due to hypothyroidism. *Metab Brain Dis*. 2010 Sep;25(3):339-54. doi: 10.1007/s11011-010-9208-8. Epub 2010 Oct 1.
- Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N, Buss C. Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience*. 2017 Feb 7;342:68-100. doi: 10.1016/j.neuroscience.2015.09.070. Epub 2015 Oct 3.
- Robichaux MA, Cowan CW. Signaling mechanisms of axon guidance and early synaptogenesis. *Curr Top Behav Neurosci*. 2014
- Shafiee SM, Vafaei AA, Rashidy-Pour A. Effects of maternal hypothyroidism during pregnancy on learning, memory and hippocampal BDNF in rat pups: Beneficial effects of exercise. *Neuroscience*. 2016 Aug 4;329:151-61. doi: 10.1016/j.neuroscience.2016.04.048. Epub 2016 May 12.
- OECD no. 207: New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. <http://www.oecd-ilibrary.org/docserver/download/9717091e.pdf?expires=1517915713&id=id&accname=guest&checksum=B30ECD7FE792AD539750CCE7AC8B15E5>.

Annex 3: Written response from the authors in preparation for the end of review Teleconference

AOP 54: Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment

Alexandra Rolaki, Francesca Pistollato, Sharon Munn and Anna Bal-Price* (*corresponding author: anna.price@ec.europa.eu)

The text marked in yellow will be considered to be introduced in the updated version of this AOP. Responses to reviewers are written in blue.

Reviewer #1

Scientific quality:

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

In general much relevant literature has been incorporated into the descriptions of the shown KE and KERs. I do however find that a few issues would benefit from some revision.

1) In the last sentence of the **abstract** it is stated that “Perchlorate, which is the most potent inhibitor of NIS, has been associated with reduced TH production and **also with cognitive deficits in animals...**”. I however do not find that there is very good data to support this argument. A literature search in PubMed (February 2018), revealed that neurobehavioural effects after perinatal perchlorate exposure have been investigated in the following three *in vivo* studies (York et al 2004 & 2005, vanWijk et al 2008, Gilbert & Sui). In my opinion none of the obtained results support this conclusion very well.

- Gilbert ME, Sui L. Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat. *Environ Health Perspect.* 2008 Jun;116(6):752-60
- van Wijk N, Rijntjes E, van de Heijning BJ. Perinatal and chronic hypothyroidism impair behavioural development in male and female rats. *Exp Physiol.* 2008 Nov;93(11):1199-209
- York RG, Barnett J, Girard MF, Mattie DR, Bekkedal MV, Garman RH, Strawson JS. Refining the effects observed in a developmental neurobehavioral study of ammonium perchlorate administered orally in drinking water to rats. II. Behavioral and neurodevelopment effects. *Int J Toxicol.* 2005 Nov-Dec;24(6):451-67
- York RG, Barnett J Jr, Brown WR, Garman RH, Mattie DR, Dodd D. A rat neurodevelopmental evaluation of offspring, including evaluation of adult and neonatal thyroid, from mothers treated with ammonium perchlorate in drinking water. *Int J Toxicol.* 2004 May-Jun;23(3):191-214.

York et al. (2004) conclude that: “There were no behavioral effects in the offspring exposed as high as 10.0 mg/kg-day as evaluated by passive avoidance, swimming water maze, motor activity, and auditory startle.” In their re-evaluation of the data (York et al. 2005) the authors again conclude that:” The behavioral testing suggests prenatal exposure to ammonium perchlorate does not affect the development of gross motor movements in the pups”, and Gilbert & Sui (2008) write in their abstract that: ”Perchlorate did not impair motor activity, spatial learning, or fear conditioning”.

vanWijk et al (2008) was the only publication to show small effects on neurobehavioural development in perinatally perchlorate treated animals. This paper showed delayed neuro-motor skill during early postnatal development (measured as grip-test and balance beam). This effect was however most probably caused by low postnatal body weight, as the neuro-motor skills were no longer observed later in life, when the animals were no longer low in body weight. The authors found no effect on locomotor activity (open field) in either sex, but did find small but significant adverse effects in learning and memory test (Morris maze) in female offspring (but not in males). However the study was performed with only one

dose group exposed to perchlorate only during development, and the behavioural assessments were performed using a group size of 5-8, making the reliability of this study, quite limited.

In some of these studies, other neurological endpoints than behavior, were adversely affected. There was an apparent increase in the thickness of the corpus callosum in day 12 pups (York et al 2005) and Gilbert & Sui (2008) found dose-dependent deficits in hippocampal synaptic function.

Together, these findings indicate that brain development may indeed be affected by perinatal perchlorate exposure, but all in all, there are no consistent data showing adverse behavioral effects in the offspring (at doses causing altered thyroid hormone levels during development). A discussion of this point is in my opinion detrimental, and is presently completely missing from this AOP. It would be especially beneficial in the WoE discussion of KER (Inhibition of NIS leads to impairment in learning and memory) on page 47. Unfortunately here, only data from the van Wijk et al (2008) study are included and none of the limitations of this study are mentioned. This issue in my opinion needs some revision.

Response: following reviewer's concerns, and taking into account the chemical agnostic nature of AOPs, we have modified *Abstract* accordingly by removing the text referring to specific chemicals and adding two last sentences: The overall weight of evidence for this AOP is strong. The function of NIS and its essentiality for TH synthesis is well known across species, however, quantitative information of KERs is limited.

Furthermore, we commented on additional studies including recently published ones describing the effects of decreased NIS activity on thyroidal status and their possible effects on learning and memory impairment. We added this text/references under 'Weight of Evidence' (in indirect KER 1503: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory):

- **Kosugi et al. 1998; Ferrandino et al. 2017**: Three Japanese children inherited two NIS mutations (V59E and T354P) from their healthy mother and father, respectively. V59E NIS was reported to exhibit as much as 30% of the activity of wild-type NIS (Fujiwara et al. 2000). The T354P and V59E NIS mutant proteins, when expressed in COS7 cells, were both trafficked to the cell surface, but totally inactive. The three siblings displayed different degrees of mental retardation, including heavy learning and memory deficits. The oldest one was nursed for longer than the second oldest, and evinced a less severe cognitive deficit. The youngest was not nursed, and displayed a more severe cognitive deficit than either of her siblings. It was discovered that the mother was addicted to laminaria, an alga extremely rich in I⁻ (Ferrandino et al. 2017). These studies have been also cited in support of Essentiality for KE (MIE).

- **Babu et al. 2011**: in this in vivo study 50-day-old female rats weighing 120–150 g were switched to a low iodine diet (LID) and given 1% KClO₄ (NIS inhibitor) in drinking water for 10 days. Animals were then separated into an iodine sufficient groups (or euthyroid) and a low iodine diet (or hypothyroxinemic) group (0.005% KClO₄) and kept on above diet regimen for 3 months. Based on the hormonal estimations and urinary iodine, female rats were further divided into euthyroid and hypothyroxinemic and were mated with normal males. In a separate group of age-matched female rats, hypothyroidism was induced in rats by giving MMI (0.025% wt/vol) in drinking water to the pregnant rats from gestational day 8 and continued thereafter until sacrifice of pups born to these dams (hypothyroid group).

Data showed a significant reduction in total serum T4 and T3 levels of rat pups administered with MMI compared to euthyroid controls (3-fold decrease of T3 vs ctr and 7-fold decrease of T4 at P16). Hypothyroxinemic pups (on low iodine diet and KClO₄) showed a reduction in serum T4 (~ 70% decrease of T4 vs ctr) but not in T3, which was increased compared to euthyroid levels at P16 (~ 40% increase of T3 vs ctr). Even in the presence of elevated circulating T3 levels, hypothyroxinemic pups showed significantly impairment of TH responsiveness in developing rat neocortex.

Both hypothyroid (MMI) and hypothyroxinemic (KClO₄) pups demonstrated a significant increase in D2 levels compared to controls (~ 11 fold in hypothyroidism, and ~ 4 fold in hypothyroxinemia). The expression of D3 mRNA was also decreased significantly (by ~ 3.3 fold in hypothyroidism and ~ 3 fold in hypothyroxinemic group compared to controls), whilst MCT8 and TH nuclear receptors α 1 and β 1

expression did not change. Additionally, myelin basic protein (MBP) protein levels and gene were decreased in both groups (for MBP gene: by ~ 60% and ~ 70% respectively in hypothyroidism and hypothyroxinemic groups vs Ctr). Moreover, increased number of apoptotic neurons was found evenly distributed in all the layers of the neocortex under both hypothyroxinemic and hypothyroid conditions. As stated in this study, altogether these data suggest that hypothyroxinemia induced by low iodine diet and KClO₄ may lead to learning and memory impairment in this model. However, memory or cognitive tests were not assessed in this study.

- **Buras et al. 2014:** in this in vivo study 9-10 week old mice were administered with drinking water containing 0.05% MMI and 1% KClO₄ for 4 weeks to render them hypothyroid. After 4 weeks, the hypothyroid group was further divided into 3 groups: the hypothyroid (0.05% MMI + 1% KClO₄), T3 (0.05% MMI + 1% KClO₄ + T3 (0.5 µg/ml) in drinking water) and T4 (0.05% MMI + 1% KClO₄ + T4 (5 µg/ml) in drinking water) groups for weeks 5 and 6. T3 serum levels were decreased by ~ 40% in hypothyroid group vs Ctr, and T4 was totally depleted in hypothyroid group vs Ctr. Several tests were performed to evaluate fear-anxiety behaviour. In the elevated plus maze, the hypothyroid mice showed significantly lower distance and time in the open arms than the T3-treated group (~ 50% for both parameters) than the euthyroid controls. The hypothyroid group also showed greater distance and time in the closed arms (~ 10% and 20% more than Ctr respectively for distance and time scores) than the T3-treated group. Administration of T3 and T4 rescued these effects. Moreover, hypothyroid mice froze more than Ctr (~ 35% more) and T3 and T4 treatments reversed this effect.

- **Navarro et al. 2015:** in this in vivo study 0.02% MMI and 1% KClO₄ were added to the drinking water in rats starting at embryonic day 10 (E10, developmental hypothyroidism) and E21 (early postnatal hypothyroidism) until day of sacrifice at PND 50. Behavior was studied using the acoustic prepulse inhibition (somatosensory attention) and the elevated plus-maze (anxiety-like assessment) tests. Total plasmatic T4 levels of both E10 (1.86 ng/ml) and E21 (1.08 ng/ml) pups were significantly lower than those of Ctr (36.29 ng/ml) pups. Total plasmatic T3 levels of E10 (0.10 ng/ml) and E21 (0.10 ng/ml) were significantly lower than in Ctr (0.45 ng/ml) pups. E10 and E21 treated pups showed abnormal laminar organization of the hippocampus, critical brain structure for learning and memory processes. The distribution, density and size of VGluT1 and VGAT boutons in the hippocampus and somatosensory cortex was abnormal in hypothyroid pups (in both groups) and these changes correlated with behavioral changes: prepulse inhibition of the startle response amplitude was reduced (23.3% in E10, 43.0% in E21 and 79.0% in Ctr pups), indicating severe pre-attention deficit in treated pups, while the percentage of time spent in open arms increased (57.0% time spent in open arms in E21 and 81.1% in E10 pups, vs 17.1% Ctr pups, indicative of increased anxiety).

- **Vasilopoulou et al. 2016:** this in vivo study investigated the effects of adult onset hypothyroidism (induced by administration of 1% w/v KClO₄ in their drinking water for 8 weeks in adult male Balb/cJ mice) on acetylcholinesterase (AChE) activity and on related behavioral parameters. They found that adult onset hypothyroidism (TH levels were not measured in this study) caused decrease of memory and increased fear/anxiety (i.e., 51% decrease of time spent in open arms / [times spent in open + closed arms], 47% decrease of the number of entries into the open arms of the apparatus, and 42% decrease in the total number of arm entries), and activity of both isoforms of AChE was reduced in all examined brain regions. **done**

Additionally, as already reported under indirect KER 1503, an epidemiological study by [Taylor et al. \(2014\)](#) supports the link between perchlorate exposure during pregnancy, maternal TH reduction and cognitive impairment in children. In this study, first trimester maternal perchlorate levels in the highest 10% of the study population (i.e., highest exposure to perchlorate in mothers who resulted hypothyroid/hypothyroxinemic during pregnancy) were associated with increased odds of offspring IQ in the lowest 10% at 3 years of age, as shown in 487 mother-child pairs.

Under '**Uncertainties or Inconsistencies**' in indirect KER 1503: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory), we took into account the studies indicated by the reviewer (i.e., [Gilbert et al. 2008](#); [York et al. 2004](#); [York et al. 2005](#)), which did not show effects of perchlorate

exposure on cognitive functions. We also commented on the possible limitations of van Wijk et al. 2008 study (in the same section: 'Uncertainties or Inconsistencies' in indirect KER 1503):

"It should be noted that van Wijk et al. 2008 study was performed with only one dose group exposed to perchlorate during development, and the behavioural assessments were performed using a limited group size of 5-8, possibly reducing the reliability of this study. In general, chronic hypothyroidism effects on development were more pronounced than the effects of perinatal hypothyroidism, suggesting that functional alterations occurring as a consequence of hypothyroidism may be partly reversible depending on developmental stage of the deficiency.

Opposite, other in vivo studies do not support associations between perinatal perchlorate exposure and neurobehavioural effects. For example, York et al. (2004) could not observe meaningful behavioral effects in rat offspring exposed as high as 10.0 mg/kg/day, as evaluated by passive avoidance, swimming water maze, motor activity, and auditory startle. In their re-evaluation of the data (York et al. 2005), authors concluded that rat pups exposed to perchlorate both during pregnancy and after 10 days of lactation, despite showing alterations of neurohistopathological features, did not show altered development of gross motor movements. Moreover, Gilbert and Sui (2008) found that adult male offspring born from rat dams exposed to 0, 30, 300, or 1,000 ppm perchlorate in drinking water from gestational day 6 until weaning, underwent reduction of T3 (10–14% reduction) and T4 (~ 9–20% reduction) reduction on postnatal day 21 (at the highest perchlorate dose), significant reductions in baseline synaptic transmission (~ 20% increase in excitatory postsynaptic potential slope amplitude), but without changes of motor activity, spatial learning, or fear conditioning."

Finally, to make stronger rationale for indirect KER 1503 (Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory), we also added under 'Biological Plausibility' the following text:

"During pre- and perinatal development, disruption of TH signaling leads to a multitude of neurological deficits. Multiple studies have shown that TH deprivation leads to defects in learning processes (for a comprehensive review, see Raymaekers and Darras, 2017). Congenital hypothyroidism has been shown to cause selective visuocognitive malfunctions, a lower IQ even in young adults (Oerbeck et al., 2003; Simic et al., 2013; Wheeler et al., 2012; Willoughby et al., 2014). On the other hand, adult-onset hyperthyroidism has been associated with a decrease in signal activity between the hippocampus and other cortical regions (Zhang et al., 2014), hyperactivity, attention deficits and changes in anxiety state (Raymaekers and Darras, 2017), which could impact learning potential."

References (to be added under KER 1503):

Babu S, Sinha RA, Mohan V, Rao G, Pal A, Pathak A, Singh M, Godbole MM (2011). Effect of hypothyroxinemia on thyroid hormone responsiveness and action during rat postnatal neocortical development. *Exp Neurol*. Mar;228(1):91-8.

Brent GA (2014). Perchlorate Exposure in Pregnancy and Cognitive Outcomes in Children: It's Not Your Mother's Thyroid. *J Clin Endocrinol Metab*. Nov; 99(11): 4066–4068.

Buras A, Battle L, Landers E, Nguyen T, Vasudevan N (2014). Thyroid hormones regulate anxiety in the male mouse. *Horm Behav*. Feb;65(2):88-96.

Ferrandino G, Kaspari RR, Reyna-Neyra A, Boutagy NE, Sinusas AJ, Carrasco N (2017). An extremely high dietary iodide supply forestalls severe hypothyroidism in Na⁺/I⁻ symporter (NIS) knockout mice. *Sci Rep*. 2017 Jul 13;7(1):5329.

Fujiwara H, Tatsumi K, Tanaka S, Kimura M, Nose O, Amino N (2000). A novel hV59E missense mutation in the sodium iodide symporter gene in a family with iodide transport defect. *Thyroid* 10:471–474.

Kosugi S, Inoue S, Matsuda A, Jhiang SM (1998). Novel, missense and loss-of-function mutations in the sodium/iodide symporter gene causing iodide transport defect in three Japanese patients. *J Clin Endocrinol Metab*. Sep;83(9):3373-6.

Gilbert ME, Sui L (2008). Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat. *Environ Health Perspect*. Jun;116(6):752-60.

- Navarro D, Alvarado M, Navarrete F, Giner M, Obregon MJ, Manzanares J, Berbel P (2015). Gestational and early postnatal hypothyroidism alters VGluT1 and VGAT bouton distribution in the neocortex and hippocampus, and behavior in rats. *Front Neuroanat.* Feb 17;9:9.
- Oerbeck B, Sundet K, Kase BF, Heyerdahl S (2003). Congenital hypothyroidism: influence of disease severity and l-thyroxine treatment on intellectual, motor, and school-associated outcomes in young adults. *Pediatrics*, 112, pp. 923-930.
- Pearce EN, Lazarus JH, Smyth PP, et al. Perchlorate and thiocyanate exposure and thyroid function in first-trimester pregnant women. (2010). *J Clin Endocrinol Metab.* 95:3207–3215.
- Raymaekers SR, Darras VM (2017). Thyroid hormones and learning-associated neuroplasticity. *Gen Comp Endocrinol.* Jun 1;247:26-33.
- Simic N, Khan S, Rovet J (2013). Visuospatial, visuoperceptual, and visuoconstructive abilities in congenital hypothyroidism. *J. Int. Neuropsychol. Soc.*, 19, pp. 1119-1127.
- Vasilopoulou CG, Constantinou C, Giannakopoulou D, Giompres P, Margarity M. (2016). Effect of adult onset hypothyroidism on behavioral parameters and acetylcholinesterase isoforms activity in specific brain regions of male mice. *Physiol Behav.* Oct 1;164(Pt A):284-91.
- Wheeler SM, McAndrews MP, Sheard ED, Rovet J (2012). Visuospatial associative memory and hippocampal functioning in congenital hypothyroidism. *J. Int. Neuropsychol. Soc.*, 18, pp. 49-56.
- Willoughby KA, McAndrews MP, Rovet JF (2014). Effects of maternal hypothyroidism on offspring hippocampus and memory. *Thyroid*, 24, pp. 576-584.
- York RG, Barnett J Jr, Brown WR, Garman RH, Mattie DR, Dodd D (2004). A rat neurodevelopmental evaluation of offspring, including evaluation of adult and neonatal thyroid, from mothers treated with ammonium perchlorate in drinking water. *Int J Toxicol.* May-Jun;23(3):191-214.
- York RG, Barnett J, Girard MF, Mattie DR, Bekkedal MV, Garman RH, Strawson JS (2005). Refining the effects observed in a developmental neurobehavioral study of ammonium perchlorate administered orally in drinking water to rats. II. Behavioral and neurodevelopment effects. *Int J Toxicol.* Nov-Dec;24(6):451-67.
- Zhang W, Liu X, Zhang Y, Song L, Hou J, Chen B, He M, Cai P, Lii H (2014). Disrupted functional connectivity of the hippocampus in patients with hyperthyroidism: evidence from resting-state fMRI. *Eur. J. Radiol.*, 83, pp. 1907-1913.

2) In general for many of the KE and KER descriptions, references and result description from in vitro, in vivo and epidemiology studies are mixed together, so that it is hard to discern which data is from which types of studies. A clearer division of the text into results obtained from the different types of studies would ease the reading of the AOP and improve the understanding if it. This is for instance the case on P. 26 (last line), where it is not stated which test system(s) the conclusion is based on - but this is also the case several other places throughout the AOP, for instance in the overall assessment of the AOP (page 61).

Response: The studies are cited, regardless the fact they have been conducted in vivo, in vitro or in humans, as long as they support the argument under discussion. In Weight of Evidence sections we always referred to the type of studies, describing their main outcomes.

3) I find that throughout the document there is too much repetition within some of the KE and KER description. Especially the short study descriptions are often provided both in the “weight of evidence” sections, in the “empirical support for linkage” section and in some cases again under “quantitative understanding of the linkage” (e.g. on p. 24, 27, 40, 44, 53 and other pages). In the AOP 42 this is not an issue, and I find that it would improve the present AOP considerably if this issue was revised throughout the AOP.

Response: we agree with reviewer's comment, therefore we deleted some redundant text to avoid repetitions. However, if same study(ies) was/were relevant to support more than one KE or KER description we maintained the text.

4) The discussion of human data in relation to the link between perchlorate exposure and thyroid effects is in my view not fully discussed. First mentioning of this is on page 24, mid page, last sentence - before

the “Biological Plausibility” paragraph. Here a reference is made to only one epidemiology study (Braverman et al 2006), not showing any significant link, between perchlorate exposure and TH levels. The paragraph ends with a sentence that the study is “obviously statistically underpowered”, indicating that its conclusions are not very reliable. I would suggest to include number of study participants in order to substantiate this claim, as no study description is included in this KE description under “empirical support for linkage”. Other study references investigating this issue could be provided here.

Response: Braverman et al 2006 study is only cited under under ‘Uncertainties or Inconsistencies’ in KER 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased (see text below):

"The thyroid system is quite complex and therefore some inconsistent results have been produced by recent studies. For example, it has been observed in healthy volunteers that a 6-month exposure to perchlorate at doses up to 3 mg/d (low doses) had no effect on thyroid function, including inhibition of thyroid iodide uptake as well as serum levels of thyroid hormones, TSH, and Tg (Braverman et al., 2006). However, this study was limited by the small sample size and is obviously underpowered."

Following reviewer's comment, we also added these additional studies under 'Uncertainties or Inconsistencies’ in KER 442':

"The review by Charnley (2008) examines a number of studies where association between perchlorate environmental (low) exposure and thyroid effects were analysed and many inconsistent conclusions have been drawn. For instance, no correlations were found between TH serum levels and urinary iodine concentrations among women exposed to perchlorate participating in the 2000-2001 National Health and Nutrition Examination Survey (NHANES). Available evidence does not support a causal relationship between changes in TH levels and current environmental levels of perchlorate exposure, but does support the conclusion that the US Environmental Protection Agency's reference dose (RfD) for perchlorate is conservatively health-protective. However, potential perchlorate risks are unlikely to be distinguishable from the ubiquitous background of naturally occurring substances present at much higher exposures that can affect the thyroid via the same biological mode of action as perchlorate, such as nitrate and thiocyanate. Therefore, risk management approaches that account for both aggregate and cumulative exposures and that consider the larger public health context in which exposures are occurring are desirable.

Additionally, a more comprehensive study by Pearce et al. (2010) conducted during 2002-2006 on 22,000 women at less than 16-week gestation showed that while low-level perchlorate exposure was ubiquitous in these women (with a median urinary perchlorate concentration of 5 µg/liter in the Turin cohort and 2 µg/liter in the Cardiff cohort), no associations between urine perchlorate concentrations and serum TSH or free T4 in the individual euthyroid or hypothyroid/hypothyroxinemic cohorts were found."

References (to be added under KER 442):

Charnley G. (2008) Perchlorate: overview of risks and regulation. Food Chem Toxicol. 46(7):2307-15 (Review).

Pearce EN, Lazarus JH, Smyth PP, et al. (2010). Perchlorate and thiocyanate exposure and thyroid function in first-trimester pregnant women. J Clin Endocrinol Metab. 95:3207–3215.

The discussion of this issue continues on page 27 (line 4-10) because here several other epidemiology studies on this issue are cited. However none of these have actually shown any significant relationship between perchlorate exposure and thyroid effects in humans (Tarone et al 2010, Tellez et al 2005, Pearce et al 2010, Steinmaus 2016b). So in reality all cited epidemiology studies come to the same conclusion as the Braverman et al 2006 study... A more clear WoE analysis, taking all available knowledge into account would be beneficial and a discussion of whether any human data actually show effects on TH status related to perchlorate exposure, would also be helpful to include here.

Response: we agree with the reviewer that exposure to environmental, low levels of perchlorate do not always decrease TH levels in humans. Charnley's (2008) review examines several studies pointing out a number of inconsistent conclusions regarding link between TH serum levels, urinary iodine concentrations, and environmental perchlorate exposure, as discussed above (this text has been added both under 'Uncertainties or Inconsistencies’ in KER 442’ and KER 872’).

However, these already cited studies in our AOP (Steinmaus et al., 2016b; Horton et al., 2015 and Brechner et al., 2000) (in 'Weight of Evidence' in KER 872: Thyroidal Iodide, Decreased leads to TH synthesis, Decreased) show that perchlorate exposure in humans is associated with increased TSH and/or decreased T4 level. We also added the following study to better support the link between perchlorate exposure and reduced TH levels (under 'Weight of Evidence' in KER 872: Thyroidal Iodide, Decreased leads to TH synthesis, Decreased):

- **Charatcharoenwiththaya et al. 2014:** this cross-sectional epidemiological study conducted in 200 pregnant Thai women with a gestational age of 14 weeks or less, showed that low-level exposure to perchlorate (i.e., 1.9 µg/L of urinary perchlorate) was positively associated with TSH and negatively associated with free T4 using multivariate analyses in first-trimester pregnant women. Low thiocyanate urinary levels (510.5 µg/L) were also positively associated with TSH in a subgroup of pregnant women with low iodine excretion (less than 100 µg/L).

We also added this additional *in vivo* study (in Quantitative Understanding of the Linkage, KER 872):

- **Gilbert et al. 2011:** this *in vivo* study assessed the effects of dietary iodine deficiency in the development of hypothyroidism. Female Long Evans rats were fed casein-based diets containing varying iodine (I) concentrations for 8 weeks (i.e., 975, 200, 125, 25, or 0 µg/kg of I added to the base diet to produce 5 nominal I levels, ranging from excess (treatment 1 = 1000 µg I/kg chow) up to deficient (treatment 5 = 25 µg I/kg chow)). Data showed that serum T4 was dose-dependently decreased relative to Treatment 1, with 19% and 48% declines at the two lowest I groups, while no significant changes in serum T3 or TSH were detected.

Reference(s) (to be added under KER 872):

Charnley G. (2008) Perchlorate: overview of risks and regulation. *Food Chem Toxicol.* 46(7):2307-15 (Review).

Charatcharoenwiththaya N, Ongphiphadhanakul B, Pearce EN, Somprasit C, Chanthasenanont A, He X, Chailurkit L, Braverman LE (2014). The association between perchlorate and thiocyanate exposure and thyroid function in first-trimester pregnant Thai women. *J Clin Endocrinol Metab.* Jul;99(7):2365-71.

Weight of evidence:

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

In general yes, with some exceptions, as mentioned above and below.

As also pointed out in my review of AOP 42, if indeed reduced levels of BDNF is a KE leading to impaired brain development, this should be reflected equally in both AOPs (i.e. a revision of AOP 42 is needed). On page 52, in the WoE section, under the study summaries, there is a short discussion of the relevance of BDNF, and the authors refer to five studies where no effect on BDNF mRNA or protein levels are seen. The AOP would however benefit greatly from a short discussion of whether the authors find that these studies are maybe less convincing or more poorly performed than those showing BDNF effects - in order to convince readers why BDNF levels should be a cornerstone in the AOP, relating low TH levels to altered brain development.

Response: we would need to know which studies the reviewer is referring to (page 52 in AOP 54 Snapshot does not correspond to the comment above) to further comment and discuss on these papers.

The discussion of this issue continues on page 59 (line 6 from the bottom) where the authors conclude that there are no inconsistencies in this KER. I find it difficult to assess whether this is correct and I would suggest to include a bit of discussion on how studies with KO mice or studies with compounds specifically inhibiting BDNF (but with no other effects) could be useful, in order to show the direct causal link.

Response: In support to KER 444 (i.e., T4 in neuronal tissue, Decreased leads to BDNF, Reduced), we added the following references/text under the 'Weight of Evidence' section, including the most recently published reports with some quantitative data showing a link between reduced TH levels and reduced BDNF levels (not discussed in the previous version). The below studies refer to the link between decreased TH levels in serum (not in the brain) leading to decreased BDNF levels, resulting in

impairment of learning and memory. However, some of these studies applied cerebral infusions of T3 (e.g., Sui et al. 2010 and Mokhtari et al. 2017) to assess effects on BDNF and learning and memory processes.

- **Sui et al. 2010:** in this in vivo study, T3, rT3 or vehicle were administered to young adult male rats either via systemic injection (i.e., IP administration of a single dose of 30 µg of T3/100 g body weight or the same dose of rT3 or the same volume of vehicle solution), or local brain infusion (i.e., intrahippocampal bilateral infusion (in the dorsal region) with 50 pmol T3, 50 pmol rT3 or vehicle (0.05% ethanol and saline solution), in 1 µl). Data showed that T3 administration increased reelin (~ 3-fold increase relative to the vehicle group at 24 h following T3 IP injection, and ~ 7-fold increase relative to the vehicle group after 1 h following T3 intrahippocampal injection), total BDNF (~ 10-fold increase relative to the vehicle group at 24 h after IP injection, and ~ 6-fold increase relative to the vehicle group at 12 h after intrahippocampal injection), and exon-specific BDNF mRNA expression in the hippocampus (after T3 IP injection: BDNF exon I and II transcripts was ~ 50-fold higher compared to the vehicle levels, whereas exon IV and VI transcripts were ~ 10-fold and ~ 30-fold higher respectively; after T3 intrahippocampal injection: exon I and II: 3.5 to 4-fold; exon IV: ~ 7-fold; exon VI: ~ 12-fold relative to the vehicles, respectively at 2 h to 24 h (for exon I), 1 h and 6 h (for exon II), 1 h (for exon IV), and 12 h (for exon VI) after T3 infusion).

Conversely, administration of rT3 (inactive T3 isoform) through IP injection or intrahippocampal infusion did not significantly alter the hippocampal reelin or BDNF mRNA levels (two pathways critical for learning and memory processes regulation). Reelin protein levels resulted increased upon both IP injection (2-fold increase 24 h after injection) and intrahippocampal infusion of T3 (3-fold increase 4 h after infusion). Likewise, BDNF protein levels were upregulated after IP injection (~ 2.7-fold increase 24 h after injection) and intrahippocampal infusion (~ 2.5-fold increase 12 h after infusion) of T3. Analysis of transcriptional coactivators binding (e.g., cAMP response element binding protein-binding protein (CBP), and thyroid hormone receptor associated protein 220 (TRAP 220)) and RNA polymerase II (RNA Pol II)) revealed specific patterns of associations between such transcription factors and reelin or BDNF upon T3 administration. This study suggests that hippocampal BDNF mRNA and protein expression is under T3 regulation.

- **Blanco et al. 2013:** in this in vivo study rat dams were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. Data showed that transmission of maternal accumulated BDE-99 through placenta and breast milk caused a decrease of serum levels of T3 (by 13 ± 9% in the 2 mg/kg/day group), T4 (by 25 ± 13% in the 2 mg/kg/day group) and free-T4 (by up to 17 ± 9% in the 2 mg/kg/day group), causing downregulation of BDNF gene expression in the hippocampus of pups (by 32 ± 14% in the 2 mg/kg/day group). On the contrary, the expression of other TRs isoforms did not change in both cortex and hippocampus. Moreover, BDE-99 produced a delay in the spatial learning task in the water maze test (i.e., longer latency in reaching the platform at the highest BDE-99 dose vs control group), and a dose-response anxiolytic effect as revealed by the open-field test.

- **Abdelhaffez and Hassan, 2013:** in this in vivo study rat dams were exposed to MMI (TPO inhibitor) to induce hypothyroidism. Pups showed a decrease of plasma free T3, free T4, and growth hormone (GH), whilst plasma TSH was significantly increased. BDNF level was significantly decreased in both the hippocampus and cerebellum of rat pups.

- **Shafiee et al., 2016:** in this in vivo study rat dams were exposed to PTU (100 mg/L in drinking water) from embryonic day 6 to their PND 21. For 14 days (from PND 31 to 44), the rat pups were trained with either the mild treadmill exercise (TE) or the voluntary wheel exercise (VE). On PND 45-52, a water maze was used for testing their learning and memory ability. Hippocampal BDNF levels were assessed one day later. Data showed that pups exposed to PTU underwent a reduction of T4 levels (~ 70%) and an increase of TSH levels (~ 80%) at PND21. A reduction of hippocampal BDNF levels (~ 7-8% reduction comparing sedentary hypothyroid vs sedentary control pups) was observed in the treatment group. The conclusion of this quantitative study is that hypothyroidism during the foetal period and the early postnatal period is associated with the impairment of spatial learning and memory (e.g., ~ 55-60%

increase of platform location latency in both sedentary hypothyroid male and female rats), and reduced hippocampal BDNF levels in both male and female rat offspring. Importantly, physical exercises (both VE and TE) significantly increased BDNF levels in both male and female hypothyroid animals (by ~ 2-3 percentage points) and improved learning and memory skills. Authors concluded that: "*These findings suggest that the increase in BDNF levels following a period of physical activity in hypothyroid rat pups is an important mechanism by which exercise alleviates the learning and memory deficits induced by hypothyroidism*".

- **Shi et al. 2017**: in this in vivo study rat dams were randomly treated with BDE-209 (100, 300, and 900 mg/kg body weight) or corn oil by gavage on gestational days 6 to 20. Blood was obtained through heart puncture for TH analysis in male rat offspring on PND 60. Data indicated that BDNF protein levels in the hippocampus decreased by 13% and 33% respectively in the 300 mg/kg and 900 mg/kg dose group. Total T4 levels and free T4 levels were significantly decreased in the BDE-209 treated-group (900 mg/kg, 300 mg/kg), and total T3 levels in 300 mg/kg group were also significantly decreased compared ctr (no significant difference was observed in 100 mg/kg group). In this study, decreased BDNF levels are well correlated with decrease of total and free T4 levels occurring upon exposure to BDE-209.

- **Mokhtari et al. 2017**: in this in vivo study rats underwent transient middle cerebral artery occlusion (tMCAo) to induce ischemic brain stroke. Rats were randomly divided in four groups: Co (control), Sh (sham), tMCAo and tMCAo + T3 (intracerebroventricular injection of T3 at 25 ug/kg body administered 24 after reperfusion). T3 significantly improved the learning and memory compared with tMCAo group, as shown by Morris water maze test. Step-through latency significantly increased in the T3 group compared with tMCAo group. Moreover, BDNF mRNA and protein levels were decreased in the tMCAo compared with Co and Sh group (~ 15% decrease of protein and ~ 20% decrease of mRNA vs Co or Sh), and addition of T3 increased BDNF mRNA and proteins compared to Co, Sh and tMCAo groups (~ 94% increase of protein and ~ 750% increase of mRNA comparing tMCAo + T3 vs tMCAo). This study points out again that BDNF levels are under the control of T3.

- **Sabbaghziarani et al. 2017**: similar to previous study from Mokhtari et al. (2017), here cerebral ischemia was induced by MCAo in male Wistar rats; a group of rats was also injected with T3 (25 µg/kg, IV injection) at 24 hours after ischemia. BDNF gene and protein levels (along with nestin and Sox2) were increased upon T3 treatment vs ischemic group. T3 treated rats also showed higher levels of serum T3 and T4, and lower levels of TSH vs ischemic group 4 days post ischemia induction. These data globally indicate that brain increased T3 levels increase BDNF expression and protein levels.

- **Kawahori et al. 2018**: in this in vivo study rat dams were administered with MMI (0.025% w/v) from 2 weeks prior to conception until delivery, which induced mild maternal hypothyroxinemia during pregnancy, comparing MMI and control offspring at day 28 and day 70 after birth. MMI-exposed pups showed an impaired learning capacity in the behavior tests. Hippocampal steady-state Bdnf exon IV (responsible for neural activity-dependent Bdnf gene expression) expression was lower in MMI group than in Ctr at day 28, while at day 70, hippocampal Bdnf exon IV expression at the basal level was comparable between the two groups. Additionally, persistent DNA hypermethylation was found in the promoter region of Bdnf exon IV in the hippocampus of MMI group vs ctr, which may be responsible for the decrease of Bdnf exon IV expression in the treated group.

References (inserted in KER 444):

Abedelhaffez AS, Hassan A (2013). Brain derived neurotrophic factor and oxidative stress index in pups with developmental hypothyroidism: neuroprotective effects of selenium. *Acta Physiol Hung.* Jun;100(2):197-210.

Blanco J, Mulero M, Heredia L, Pujol A, Domingo JL, Sánchez DJ (2013). Perinatal exposure to BDE-99 causes learning disorders and decreases serum thyroid hormone levels and BDNF gene expression in hippocampus in rat offspring. *Toxicology.* Jun 7;308:122-8.

Kawahori K, Hashimoto K, Yuan X, Tsujimoto K, Hanzawa N, Hamaguchi M, Kase S, Fujita K, Tagawa K, Okazawa H, Nakajima Y, Shibusawa N, Yamada M, Ogawa Y (2018). Mild Maternal

Hypothyroxinemia During Pregnancy Induces Persistent DNA Hypermethylation in the Hippocampal Brain-Derived Neurotrophic Factor Gene in Mouse Offspring. *Thyroid*. Mar;28(3):395-406.

Mokhtari T, Akbari M, Malek F, Kashani IR, Rastegar T, Noorbakhsh F, Ghazi-Khansari M, Attari F, Hassanzadeh G (2017). Improvement of memory and learning by intracerebroventricular microinjection of T3 in rat model of ischemic brain stroke mediated by upregulation of BDNF and GDNF in CA1 hippocampal region. *Daru*. Feb 15;25(1):4.

Sabbaghziarani F, Mortezaee K, Akbari M, Kashani IR, Soleimani M, Hassanzadeh G, Zendedel A (2017). Stimulation of neurotrophic factors and inhibition of proinflammatory cytokines by exogenous application of triiodothyronine in the rat model of ischemic stroke. *Cell Biochem Funct*. Jan;35(1):50-55.

Shi R, Xie X, Gao Y, Zhou YJ, Zhang Y, Chen LM, Tian Y (2017). [The effects of prenatal exposure to brominated diphenyl ethers-209 to the influence of male offspring rats hippocampus BDNF protein expression and its mechanism of action]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*. Sep 20;35(9):652-655.

Sui L, Ren WW, Li BM (2010). Administration of thyroid hormone increases reelin and brain-derived neurotrophic factor expression in rat hippocampus in vivo. *Brain Res*. Feb 8;1313:9-24.

Additionally, to support indirect KER 1507 (BDNF, Reduced leads to Impairment, Learning and memory), we added the following studies under the 'Weight of Evidence' section:

- **Bekinschtein et al. 2008**: in this in vivo study, the protein synthesis inhibitor anisomycin (Ani; 80 µg/0.8 µl per side) was injected in the dorsal hippocampus of Male Wistar rats (2.5 months) 12 h after inhibitory avoidance (IA) training (i.e., using a strong foot shock, which generates a persistent LTM), which causes a selective deficit in memory retention 7 days, but not 2 days, after training. Human recombinant BDNF (hrBDNF, 0.25 µg/0.8 µl per side) or vehicle (Veh) was delivered 15 min after Ani infusion into the hippocampus. hrBDNF completely rescued long-term memories (LTM) at 7 days after training caused by Ani given at 12 h after training. Additionally, infusion of BDNF antisense oligonucleotides (i.e., BDNF ASO, which blocks the expression of BDNF 12 h after training) into the dorsal hippocampus 10 h after training, was found to impair persistence (a characteristic feature of LTM), but not formation of IA LTM (as compared with BDNF missense oligonucleotide). This indicates that BDNF during the late posttraining critical time period is not only required but sufficient for persistence of LTM storage. This study also supports essentiality of this KE (i.e., decreased BDNF).

- **Alonso et al. 2002**: in this study the role of BDNF in both short and long term memories (STM and LTM) formation of a hippocampal-dependent one-trial fear-motivated learning task was examined in male Wistar rats (2–3 months). IA training was found associated with a rapid and transient increase in BDNF mRNA expression (by 90%, 1 hr after IA training) in the hippocampus. Bilateral infusions of function-blocking anti-BDNF antibody (0.5 µg/side) into the CA1 region of the dorsal hippocampus decreased ERK2 activation, and blocked STM formation. On the contrary, intrahippocampal administration of rhBDNF (0.25 µg/side) increased ERK1/2 activation and facilitated STM. These results strongly indicate that endogenous BDNF is required for both STM and LTM formation of an IA learning. This study also supports essentiality of this KE (i.e., decreased BDNF).

- **Blanco et al. 2013**: in this in vivo study rat dams were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. Data showed that transmission of maternal accumulated BDE-99 through placenta and breast milk caused a decrease of serum levels of T3 (by 13 ± 9% in the 2 mg/kg/day group), T4 (by 25 ± 13% in the 2 mg/kg/day group) and a decrease of free-T4 (by up to 17 ± 9% in the 2 mg/kg/day group), causing downregulation of BDNF gene expression in the hippocampus of pups (by 32 ± 14% in the 2 mg/kg/day group). Moreover, BDE-99 produced a delay in the spatial learning task in the water maze test (i.e., longer latency in reaching the platform at the highest BDE-99 dose vs control group), and a dose-response anxiolytic effect as revealed by the open-field test.

To make stronger rationale for indirect KER 1507 (BDNF, Reduced leads to Impairment, Learning and memory), under 'Biological Plausibility' we added the following text:

"BDNF protein is synthesized as a precursor (pre-proBDNF), resulting after cleavage in a 32-kDa proBDNF protein. ProBDNF is either proteolytically cleaved intracellularly by enzymes like furin or pro-

convertases and secreted as the 14 kDa mature BDNF (mBDNF), or secreted as proBDNF and then cleaved by extracellular proteases, such as metalloproteinases and plasmin, to mBDNF (see Lessmann et al., 2003). Both proBDNF and mBDNF are preferentially sorted and packaged into vesicles of the activity-regulated secretory pathway. ProBDNF is not an inactive precursor of BDNF; it is released in the immature and mature CNS in an activity dependent manner (for a comprehensive review on the role of BDNF in learning and memory, see Cunha et al. 2010). The intracellular localization of BDNF is predominantly somatodendritic, but it is also enriched in the dendrites. BDNF can activate several signalling pathways (e.g., ERK (Orban et al., 1999; Sweatt, 2004; Thomas and Huganir, 2004), PI3K–Akt (Lin et al., 2001), CREB (Barco et al., 2003)) that may regulate downstream cellular effects necessary for synaptic plasticity and memory formation. The role of BDNF in synaptogenesis and neuronal network functions, which represent the KEs before the AO (decrease of learning and memory), was already described in other three AOPs (i.e., 13, 48 and 12) already endorsed by OECD. Importantly, reduced levels of BDNF have been reported as a consequence of decreased TH levels, playing a crucial role in neuroplasticity, one of the fundamental processes in learning and memory (Chakraborty et al., 2012; Gilbert and Lasley, 2013). In line with this, BDNF-mediated stimulation of both hippocampal neurogenesis and inhibition of hippocampal apoptosis can recover spatial memory deficits triggered by developmental hypothyroidism in rats (Shafiee et al., 2016; Shin et al., 2013)."

References (inserted in KER 1507):

Alonso M, Vianna MR, Depino AM, Mello e Souza T, Pereira P, Szapiro G, Viola H, Pitossi F, Izquierdo I, Medina JH (2002). BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. *Hippocampus*. 12(4):551-60.

Barco A, Pittenger C, Kandel ER (2003). CREB, memory enhancement and the treatment of memory disorders: promises, pitfalls and prospects. *Expert Opin Ther Targets*. Feb; 7(1):101-14.

Bekinschtein P, Cammarota M, Katche C, Slipczuk L, Rossato JI, Goldin A, Izquierdo I, Medina JH (2008). BDNF is essential to promote persistence of long-term memory storage. *Proc Natl Acad Sci U S A*. Feb 19;105(7):2711-6.

Blanco J, Mulero M, Heredia L, Pujol A, Domingo JL, Sánchez DJ (2013). Perinatal exposure to BDE-99 causes learning disorders and decreases serum thyroid hormone levels and BDNF gene expression in hippocampus in rat offspring. *Toxicology*. Jun 7;308:122-8.

Cunha C, Brambilla R, Thomas KL (2010). A simple role for BDNF in learning and memory? *Front Mol Neurosci*. Feb 9;3:1.

Gilbert ME, Lasley SM (2013). Developmental thyroid hormone insufficiency and brain development: a role for brain-derived neurotrophic factor (BDNF)? *Neuroscience*, 239, pp. 253-270.

Lessmann V, Gottmann K, Malsangio M (2003). Neurotrophin secretion: current facts and future prospects. *Prog Neurobiol*. Apr; 69(5):341-74.

Lin CH, Yeh SH, Lin CH, Lu KT, Leu TH, Chang WC, Gean PW (2001). A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. *Neuron*. Sep 13; 31(5):841-51.

Orban PC, Chapman PF, Brambilla R (1999). Is the Ras-MAPK signalling pathway necessary for long-term memory formation? *Trends Neurosci*. Jan; 22(1):38-44.

Shin MS, Ko IG, Kim SE, Kim BK, Kim TS, Lee SH, Hwang DS, Kim CJ, Park JK, Lim BV (2013). Treadmill exercise ameliorates symptoms of methimazole-induced hypothyroidism through enhancing neurogenesis and suppressing apoptosis in the hippocampus of rat pups. *Int J Dev Neurosci*. May;31(3):214-23.

Sweatt JD (2004). Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr Opin Neurobiol*. Jun; 14(3):311-7.

Thomas GM, Huganir RL (2004). MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci*. Mar; 5(3):173-83.

On page 47, in the WoE discussion of KER 1503 (Inhibition of NIS leads to impairment in learning and memory) the data chosen to support the KER are in my opinion not very convincing. The AOP authors

include two animal studies investigating the effect of perinatal BPA exposure on cognitive function (Wang et al 2016 & Jang et al 2012). And while BPA may act as a NIS inhibitor in vitro, little evidence points to this compound causing marked T4 reductions in vivo. To me, it therefore seems much more plausible that the effects of BPA on cognitive function (shown in these two and a large number of other in vivo studies) was probably not NIS (T4) mediated, but possibly caused by the estrogenic or other effects of BPA.

Response: some in vivo studies have also linked BPA exposure to hypothyroidism, although without proving direct inhibitory effects of BPA on NIS activity. We commented on this at the beginning of 'Empirical Evidence' (in indirect KER 1503: Inhibition of NIS leads to impairment in learning and memory):

"BPA exposure has been also associated with hypothyroidism (i.e., decreased of free T3 and free T4, increase of TSH plasma levels, perturbation of thyroid gland morphological structure and thyroid cell function) in humans (i.e., inverse relationships between urinary BPA and total T4 and TSH) (Meeker and Ferguson, 2011), in young rats breast-fed from mothers treated with BPA (Mahmoudi et al. 2018), and in pregnant ewes and their newborn lambs (i.e., decrease of total T4 in BPA-treated pregnant ewes and in the cord and the jugular blood of their newborns (30% decrease), and of plasma free T4 levels in the jugular blood of the newborns) (Viguié et al. 2013)."

References (inserted in KER 1503):

Mahmoudi A, Ghorbel H, Feki I, Bouallagui Z, Guermazi F, Ayadi L, Sayadi S (2018). Oleuropein and hydroxytyrosol protect rats' pups against bisphenol A induced hypothyroidism. *Biomed Pharmacother.* Apr 27;103:1115-1126.

Meeker JD, Ferguson KK (2011). Relationship between urinary phthalate and bisphenol A concentrations and serum thyroid measures in U.S. adults and adolescents from the National Health and Nutrition Examination Survey (NHANES) 2007-2008. *Environ Health Perspect.* Oct;119(10):1396-402.

Viguié C, Collet SH, Gayraud V, Picard-Hagen N, Puel S, Roques BB, Toutain PL, Lacroix MZ (2013). Maternal and fetal exposure to bisphenol a is associated with alterations of thyroid function in pregnant ewes and their newborn lambs. *Endocrinology.* Jan;154(1):521-8.

Then some epidemiology studies that are cited, relating exposure levels of perchlorate and PBDEs in pregnant women, to cognitive deficits in their children (Taylor et al 2014; Chen et al 2014; Roze et al 2009). But does the Taylor et al 2014 study actually investigate whether the perchlorate exposed mothers were hypothyroxinemic? If this is not shown, the observed IQ reductions could be related to concomitant co-exposures to other chemicals in these women.

Response: To take into account the possible uncertainties with regards to perchlorate exposure and maternal thyroid functions in Taylor et al. 2004 study, we added this text under 'Uncertainties or Inconsistencies' (in indirect KER 1503: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory):

"Taylor et al. 2004 (CATS study) identified 1050 pregnant women with hypothyroidism or hypothyroxinemia; half were in the immediate T4 treatment group, and half were in the group tested and treated after pregnancy. 487 (46.4%) mother-child pairs completed psychological testing and urinary iodine and perchlorate measurements. Therefore, the 487 women-child pairs represent approximately two-thirds of those reported in the study of T4 treatment effects on cognitive outcome. Taking this into account, the absence of a direct effect of perchlorate on maternal thyroid function (Pearce et al. 2010), suggests that developmental effects of perchlorate may not necessarily be linked to maternal thyroid hormone levels, as commented in (Brent, 2014)."

And in the Chen et al (2014), and Roze et al (2009) studies, exposure to PBDE could be affecting the developing nervous system through other MoA than via hypothyroidisms, for instance a direct neurotoxic MoA. Based on these studies (or at least the provided summaries) we are not able to tell whether any of the observed adverse effects on neurodevelopment are indeed caused by hypothyroidism (e.g. are due to NIS inhibition).

Response: PBDEs and their hydroxylated metabolites (OH-PBDEs) **have been shown** to bind to the serum-binding proteins transthyretin and thyroxine-binding globulin, affect deiodinases (DI 1, 2 and 3) activity, and alter TH metabolism and excretion, leading to hypothyroidism. Taking into account reviewer's concern, we added the following comment and related references ([at the beginning of 'Empirical Evidence'](#) (in indirect KER 1503: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory):

"PBDEs and their hydroxylated metabolites (OH-PBDEs) can bind to the serum-binding proteins transthyretin and thyroxine-binding globulin, can affect deiodinases (DI 1, 2 and 3) activity, and alter TH metabolism and excretion, leading to hypothyroidism in experimental animals (Butt et al. 2011; Marchesini et al. 2008; Meerts et al. 2000; Szabo et al. 2009; Zhou et al. 2002). Human studies also observed PBDE-associated TH disruption during pregnancy (Chevrier et al. 2010; Herbstman et al. 2008; Lin et al. 2011; Stapleton et al. 2011; Zota et al. 2011). Therefore, thyroid disruption may be a critical underlying mechanism related to the developmental neurotoxicity of PBDEs and their metabolites (Dingemans et al. 2011; Costa et al. 2008; Chen et al. 2014)."

References ([inserted in KER 1503](#)):

Butt CM, Wang D, Stapleton HM (2011). Halogenated phenolic contaminants inhibit the in vitro activity of the thyroid-regulating deiodinases in human liver. *Toxicol Sci.* Dec; 124(2):339-47.

Chevrier J, Harley KG, Bradman A, Gharbi M, Sjödin A, Eskenazi B (2010). Polybrominated diphenyl ether (PBDE) flame retardants and thyroid hormone during pregnancy. *Environ Health Perspect.* Oct; 118(10):1444-9.

Costa LG, Giordano G, Tagliaferri S, Caglieri A, Mutti A (2008). Polybrominated diphenyl ether (PBDE) flame retardants: environmental contamination, human body burden and potential adverse health effects. *Acta Biomed.* Dec;79(3):172-83.

Dingemans MM, van den Berg M, Westerink RH (2011). Neurotoxicity of brominated flame retardants: (in)direct effects of parent and hydroxylated polybrominated diphenyl ethers on the (developing) nervous system. *Environ Health Perspect.* Jul; 119(7):900-7.

Herbstman JB, Sjödin A, Apelberg BJ, Witter FR, Halden RU, Patterson DG, Panny SR, Needham LL, Goldman LR (2008). Birth delivery mode modifies the associations between prenatal polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) and neonatal thyroid hormone levels. *Environ Health Perspect.* Oct; 116(10):1376-82.

Lin SM, Chen FA, Huang YF, Hsing LL, Chen LL, Wu LS, Liu TS, Chang-Chien GP, Chen KC, Chao HR (2011). Negative associations between PBDE levels and thyroid hormones in cord blood. *Int J Hyg Environ Health.* Mar; 214(2):115-20.

Marchesini GR, Meimaridou A, Haasnoot W, Meulenberg E, Albertus F, Mizuguchi M, Takeuchi M, Irth H, Murk AJ (2008). Biosensor discovery of thyroxine transport disrupting chemicals. *Toxicol Appl Pharmacol.* Oct 1; 232(1):150-60.

Meerts IA, van Zanden JJ, Luijckx EA, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman A, Brouwer A (2000). Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol Sci.* Jul; 56(1):95-104.

Stapleton HM, Eagle S, Anthopolos R, Wolkin A, Miranda ML (2011). Associations between polybrominated diphenyl ether (PBDE) flame retardants, phenolic metabolites, and thyroid hormones during pregnancy. *Environ Health Perspect.* Oct; 119(10):1454-9.

Szabo DT, Richardson VM, Ross DG, Diliberto JJ, Kodavanti PR, Birnbaum LS (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci.* 2009 Jan; 107(1):27-39.

Zhou T, Taylor MM, DeVito MJ, Crofton KM (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci.* Mar; 66(1):105-16.

Zota AR, Park JS, Wang Y, Petreas M, Zoeller RT, Woodruff TJ (2011). Polybrominated diphenyl ethers, hydroxylated polybrominated diphenyl ethers, and measures of thyroid function in second trimester pregnant women in California. *Environ Sci Technol.* Sep 15; 45(18):7896-905.

Regulatory applicability:

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

The key question is in my opinion the quantitative link between BDNF expression and neurological impairment. On page 3, line 13, it is stated that dose-response relationships between TH levels and reduced BDNF expression in the developing brain cannot not be evaluated, as all studies have been conducted in conditions of severe maternal hypothyroidism. I however find it hard to believe that in all performed studies (using different doses of chemicals or iodine deprivation) all T4 reductions were identical, and that it would not be possible to perform some sort of no dose-reponse relationship between T4 and BDNF.

Solving this issue and of course a concomitant inclusion of BDNF measurement into more regulatory studies, would help solve this gap.

Response: in currently revised version we present additional studies, including one with semi-quantitative data (Shafiee et al., 2016), showing the empirical linkage between reduced TH levels and decreased BDNF expression and learning and memory impairments, which were reversed by upregulation of BDNF levels triggered by physical exercise. Moreover, there is strong evidence on BDNF involvement in the regulation of learning and memory processes.

All in all, this AOP is in my opinion not yet ready for regulatory use, but could maybe be combined with AOP 42, and hereafter be used for prioritization of chemicals for further in vivo testing.

Conclusion:

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

This AOP has gathered and described much relevant literature, but in my opinion there are still some major revisions needed (as stated above) prior to its proper use.

Response: we hope that the revised version of this AOP will better support its possible regulatory applicability.

Reviewer #2

Overall AOP incorporates relevant scientific literature to support the relationship between the MIE, inhibition of NIS, reduction of serum thyroid hormones, and learning and memory impairment. The evidence provided supports the biological plausibility of the pathway and associated classification of the stressors.

Observations to be considered:

MOLECULAR INITIATING EVENT

7. Age, gender, pregnancy, as well as thyroidal status and dietary iodine levels affect the impact of perchlorate stressor, inhibition of NIS and serum thyroxine levels. These factors do not seem to be prominent in the AOP. Recent studies provide additional evidence of the importance of age at exposure to stressors.

The overview for the MIE, p.2, notes the potential contradictory results from studies that showed a correlation versus no correlation between thyroid parameters and perchlorate in humans.

Horton et al. 2015 demonstrated that co-occurrence of perchlorate, nitrate, and thiocyanate alters thyroid function in pregnant women. The contradictory findings may be a result of the confounding mixtures in the environment masking the primary effect of perchlorate. PMID: PMC4641782

Taylor et al. 2014 found that higher maternal urinary perchlorate levels correlated with lower I.Q. in offspring independent of thyroxine therapy. These data suggest potentially direct impact on the development of the fetal thyroid gland. PMID: 25057878

These studies represent a body of evidence that perhaps indicates that fetal and neonatal periods of thyroid gland development are critically sensitive to NIS inhibition.

McMullen et al. 2017 that adolescents, both male and female, are more sensitive to exposure than are adults. This study clarifies that correlation between perchlorate, thiocyanate and serum T4 levels and notes again the absence of significant change in TSH. PMID: 28430972

Response: to take into account reviewer's comments, we added the following text and supporting references under 'Overview for Molecular Initiating Event' (in Event 424 (MIE): Inhibition, Na⁺/I⁻ symporter (NIS), after the sentence: "*However, there are also contradictory results from other studies that showed no correlation between thyroid parameters and perchlorate levels in humans (Pearce et al., 2010; Amitai et al., 2007; Tellez et al., 2005)*"):

"Co-occurrence of perchlorate, nitrate, and thiocyanate can alter thyroid function in pregnant women. Horton et al. (2015) have shown positive associations between the weighted sum of urinary concentrations of these three analytes and increased TSH, with perchlorate showing the largest weight in the index. Interestingly, De Groef et al. 2006 showed that nitrate and thiocyanate, acquired through drinking water or food, account for a much larger proportion of iodine uptake inhibition than perchlorate, suggesting that NIS inhibition and any potential downstream effect by perchlorate are highly dependent on the presence of other environmental NIS inhibitors and iodine intake itself (Leung et al., 2010). In particular, Tonacchera et al. (2004) showed that the relative potency of perchlorate to inhibit radioactive I⁻ uptake by NIS is 15, 30 and 240 times that of thiocyanate, iodide, and nitrate respectively on a molar concentration basis. These data are in line with earlier studies in rats (Alexander and Wolff, 1996; Greer et al. 1966). Contradictory findings in these studies may therefore be a result of the confounding mixtures in the environment, masking the primary effect of perchlorate.

Decreased iodine intake can decrease TH production, and therefore exposure to perchlorate might be particularly detrimental in iodine-deficient individuals (Leung et al. 2010). Moreover, biologically based dose-response modeling of the relationships among iodide status (e.g., dietary iodine levels), perchlorate dose, and TH production in pregnant women has shown that iodide intake has a profound effect on the likelihood that exposure to goitrogens will produce hypothyroxinemia (Lewandowski et al. 2015).

During pregnancy TH requirements increase, particularly during the first trimester (Alexander et al. 2004; Leung et al. 2010), due to higher concentrations of thyroxine-binding globulin, placental T4 inner-ring deiodination leading to the inactive reverse T3 (rT3), and transfer of small amounts of T4 to the foetus (during the first trimester foetal thyroid function is absent). Moreover, glomerular filtration rate and clearance of proteins and other molecules are both increased during pregnancy, possibly causing increased renal iodide clearance and a decreased of circulating plasma iodine (Glinoe, 1997). Thus, even though the foetal thyroid can trap iodide by about 12 week of gestation (Fisher and Klein, 1981), high concentrations of maternal perchlorate may potentially decrease thyroidal iodine available to the foetus by inhibiting placental NIS (Leung et al. 2010).

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show visual attention, visual processing and gross motor skills deficits, while if it occurs later, offspring may show subnormal visual and visuospatial skills, along with slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004).

Along this line, age and developmental stage are crucial in determining sensitivity to NIS inhibitors (e.g., perchlorate, thiocyanate, and nitrate). In this regard, McMullen et al. (2017) have shown that adolescent boys and girls, more than adults, represent vulnerable subpopulations to NIS symporter inhibitors. Altogether these studies indicate that age, gender, developmental stage, and dietary iodine levels can affect the impact of NIS inhibitors."

Moreover, under 'Overview for Molecular Initiating Event' (in Event 424: Inhibition, Na⁺/I⁻ symporter (NIS)), after the modified sentence: "**For example, perchlorate is a potent inhibitor of iodide uptake through the sodium/iodide symporter (Tonacchera et al., 2004)**", we added this text:

"Perchlorate has been detected in human breast milk ranging from 1.4 to 92.2 mg μl⁻¹ (10.5 μg I⁻¹ mean) in 18 US states (Kirk et al. 2005), and 1.3 to 411 μg I⁻¹ (9.1 μg I⁻¹ median) in the Boston area, United

States (Pearce et al. 2007). Perchlorate has also been detected in human colostrum of 46 women in the Boston area (from < 0.05 to $187.2 \mu\text{mol I}^{-1}$ (Leung et al. 2009))."

Please, note that we have extensively commented on Taylor et al. (2014) study in KER 1503 ([Inhibition, Na⁺/I⁻ symporter \(NIS\) leads to Impairment, Learning and memory](#)), adding additional text (to take into account **reviewer #1' comments**).

References (inserted in [Event 424 \(MIE\): Inhibition, Na⁺/I⁻ symporter \(NIS\)](#))

Alexander EK, Marqusee E, Lawrence J, Jarolim P, Fischer GA, Larsen PR (2004). Timing and magnitude of increases in levothyroxine requirements during pregnancy in women with hypothyroidism. *N Engl J Med*. Jul 15; 351(3):241-9.

Alexander WD and Wolff J (1996). Thyroidal iodide transport VIII, Relation between transport goitrogenic and antigoitrogenic properties of certain anions. *Endocrinology* 78 581–590.

De Groef B, Decallonne BR, Van der Geyten S, Darras VM, Bouillon R (2006). Perchlorate versus other environmental sodium/iodide symporter inhibitors: potential thyroid-related health effects. *Eur J Endocrinol*. Jul;155(1):17-25.

Fisher DA and Klein AH (1981). Thyroid development and disorders of thyroid function in the newborn. *N Engl J Med*. Mar 19; 304(12):702-12.

Glinoeir D (1997). The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev*. Jun; 18(3):404-33.

Greer MA, Stott AK & Milne KA (1966). Effect of thiocyanate, perchlorate and other anions on thyroidal iodine metabolism. *Endocrinology* 79 237–247.

Horton MK, Blount BC, Valentin-Blasini L, Wapner R, Whyatt R, Gennings C, Factor-Litvak P (2015). CO-occurring exposure to perchlorate, nitrate and thiocyanate alters thyroid function in healthy pregnant women. *Environ Res*. Nov;143(Pt A):1-9.

Kirk AB, Martinelango PK, Tian K, Dutta A, Smith EE, Dasgupta PK (2005). Perchlorate and iodide in dairy and breast milk. *Environ Sci Technol*. Apr 1; 39(7):2011-7.

Leung AM, Pearce EN, Hamilton T, He X, Pino S, Merewood A, Braverman LE (2009). Colostrum iodine and perchlorate concentrations in Boston-area women: a cross-sectional study. *Clin Endocrinol (Oxf)*. Feb; 70(2):326-30.

Leung AM, Pearce EN, Braverman LE (2010). Perchlorate, iodine and the thyroid. *Best Pract Res Clin Endocrinol Metab*. Feb;24(1):133-41.

Lewandowski TA, Peterson MK2, Charnley G (2015). Iodine supplementation and drinking-water perchlorate mitigation. *Food Chem Toxicol*. Jun;80:261-70.

McMullen J, Ghassabian A, Kohn B, Trasande L (2017). Identifying Subpopulations Vulnerable to the Thyroid-Blocking Effects of Perchlorate and Thiocyanate. *J Clin Endocrinol Metab*. Jul 1;102(7):2637-2645.

Pearce EN, Leung AM, Blount BC, Bazrafshan HR, He X, Pino S, Valentin-Blasini L, Braverman LE (2007). Breast milk iodine and perchlorate concentrations in lactating Boston-area women. *J Clin Endocrinol Metab*. May; 92(5):1673-7.

Zoeller RT, Rovet J (2004). Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. *J Neuroendocrinol*. Oct;16(10):809-18.

8. Is the Evidence considered to be only 'Moderate' for Life Stage Applicability (p.2)? Recent studies together with evidence cited throughout the key events suggest Strong Evidence particularly for Pregnancy and Birth to < 1 month.

Response: considering above added text/references under '[Overview for Molecular Initiating Event](#)' (in [Event 424 \(MIE\): Inhibition, Na⁺/I⁻ symporter \(NIS\)](#)), we changed Life Stage Applicability into **'high'**.

KEY EVENTS

The classification of the evidence in support of upstream event(s) leading (directly/indirectly) to downstream event(s) is justified adequately. AOP referencing the relationship of NIS inhibition to subsequent adverse neurodevelopmental outcomes in mammals and NIS inhibition to learning and memory impairment is strong.

KEY EVENT RELATIONSHIPS

It is helpful to have the same format for each of the relationship sections. For example, a summary of the Weight of Evidence is provided on p.24 and then citations whereas a summary/discussion is provided after citations on p. 26, 27.

Response: we harmonized the format, by providing a summary of the Weight of Evidence before the citations.

9. The statement that “The thyroid system is complex...” P. 24 cites only one study that shows no relationship of perchlorate and thyroid function. It would be more effective to cite a contrasting study e.g. McMullen et al 2017. The sentence is repeated on p.25 under uncertainties. It is more appropriate in uncertainties or inconsistencies.

Response: Following reviewer's suggestion, we cited McMullen et al. 2017, where indeed quantitative data are provided showing a relationship between perchlorate and thiocyanate, exposure and free T4 levels (under 'Weight of Evidence', in KER 305: TH synthesis, Decreased leads to T4 in serum, Decreased):

- McMullen et al. 2017: this cross-sectional analysis of data from the 2009 to 2012 National Health and Nutrition Examination Survey evaluated the exposure to perchlorate, thiocyanate, and nitrate in 3151 participants aged 12 to 80. For each log unit increase in perchlorate, free T4 decreased by 0.03 ng/dL in both the general population and in all women, and by 0.06 ng/dL in adolescent girls, corresponding to 4% and 8% decreases relative to median free T4, respectively. For each log unit increase thiocyanate, free T4 decreased by 0.07 ng/dL in adolescent boys, corresponding to a 9% decrease relative to median free T4, respectively. These data indicate that adolescent boys and girls represent vulnerable subpopulations to the thyroid-blocking effects of NIS symporter inhibitors.

Under 'uncertainties and inconsistencies', in KER 305: TH synthesis, Decreased leads to T4 in serum, Decreased, we added the following text:

The review by Charnley (2008) examines a number of studies where association between perchlorate environmental (low) exposure and thyroid effects were analysed and many inconsistent conclusions have been drawn. For instance, no correlations were found between TH serum levels and urinary iodine concentrations among women exposed to perchlorate participating in the 2000-2001 National Health and Nutrition Examination Survey (NHANES). Available evidence does not support a causal relationship between changes in TH levels and current environmental levels of perchlorate exposure, but does support the conclusion that the US Environmental Protection Agency's reference dose (RfD) for perchlorate is conservatively health-protective. However, potential perchlorate risks are unlikely to be distinguishable from the ubiquitous background of naturally occurring substances present at much higher exposures that can affect the thyroid via the same biological mode of action as perchlorate, such as nitrate and thiocyanate. Therefore, risk management approaches that account for both aggregate and cumulative exposures and that consider the larger public health context in which exposures are occurring are desirable.

References (inserted in KER 305):

Charnley G. (2008) **Perchlorate: overview of risks and regulation.** *Food Chem Toxicol.* 46(7):2307-15.

McMullen J, Ghassabian A, Kohn B, Trasande L (2017). Identifying Subpopulations Vulnerable to the Thyroid-Blocking Effects of Perchlorate and Thiocyanate. *J Clin Endocrinol Metab.* Jul 1;102(7):2637-2645.

10. The discussion of Uncertainties or Inconsistencies (p. 25 27) may be strengthened with new studies that indicate a dose relationship between perchlorate and thiocyanate exposure and thyroxine levels and outcomes.

Response: we would need to know to which papers (not included yet) the reviewer is referring to, so we could comment on them.

The section describing 'Uncertainties or Inconsistencies' should address possible inconsistencies and discrepancies in study findings. We expanded this section by adding the following text/references (under

'Uncertainties and Inconsistencies, in KER 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased):

"Decreased iodine intake can decrease TH production, and therefore exposure to perchlorate might be particularly detrimental in iodine-deficient individuals (Leung et al. 2010). Moreover, biologically based dose-response modeling of the relationships among iodide status (e.g., dietary iodine levels), perchlorate dose, and TH production in pregnant women has shown that iodide intake has a profound effect on the likelihood that exposure to goitrogens will produce hypothyroxinemia (Lewandowski et al. 2015).

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show problems in visual attention, visual processing and gross motor skills, while if it occurs later, offspring may show subnormal visual and visuospatial skills, slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004). Altogether these studies indicate that factors, such as age, gender, developmental stage, and iodide status can affect the impact of perchlorate and other NIS inhibitors. All these variables should be taken into account to explain possible inconsistencies in study findings."

References (inserted in KER 442):

Leung AM, Pearce EN, Braverman LE (2010). Perchlorate, iodine and the thyroid. *Best Pract Res Clin Endocrinol Metab.* Feb;24(1):133-41.

Lewandowski TA, Peterson MK2, Charnley G (2015). Iodine supplementation and drinking-water perchlorate mitigation. *Food Chem Toxicol.* Jun;80:261-70.

Zoeller RT, Rovet J (2004). Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. *J Neuroendocrinol.* Oct;16(10):809-18.

11. The KER of decreased T4 in serum and decreased T4 in neuronal tissue is plausible. Given the compensatory mechanisms to maintain adequate and not excessive T4/T3 in brain tissue, the degree to which decreased serum T4 directly corresponds to quantifiable decreased T4 in neuronal tissue is not yet clear. Should this be more directly stated? Nevertheless, that decreased serum TH results in lower brain TH concentrations is well established.

Response: some of these issues are already discussed in 'Biological Plausibility' in KER 312: T4 in serum, Decreased leads to T4 in neuronal tissue, Decreased). Following reviewer's indication, we added the following comment (at the end of 'Biological Plausibility'):

"Given the compensatory mechanisms to maintain adequate TH levels in brain tissue, the degree to which decreased serum T4 directly corresponds to quantifiable decreased T4 in neuronal tissue is not yet clear. However, the fact that decreased serum TH results in lower brain TH concentrations is well established."

12. Evidence for dose –dependent relationship between T3 and BDNF may be strengthened.

Sui et al. (2010) demonstrated that administration of T3 increases BDNF in rat hippocampus in vivo in a dose-dependent manner. PMID: 20018181

Mokhtari et al. (2017) established a link between T3 and upregulation of BDNF and learning and memory in an ischemic stroke model in rats. PMID: 28202057

Response: in reply to reviewer #1, we added the following references/text under the 'Weight of Evidence' section (in KER 444: T4 in neuronal tissue, Decreased leads to BDNF, Reduced), including the most recently published reports with some quantitative data (please, see pages 11-14, describing the following studies: Sui et al. 2010; Blanco et al. 2013; Abdelhaffez and Hassan, 2013; Shafiee et al., 2016; Shi et al. 2017; Mokhtari et al. 2017; Sabbaghziarani et al. 2017; Kawahori et al. 2018).

Weight of Evidence

MIE Evidence supports the classifications. Given more recent studies, the evidence in support of lifespan applicability is growing. The potential for indicating the evidence is strong rather than moderate should be considered (page 2). The scoring within the AOP for KEs, KERs is consonant with the evidence cited. Additional references such as provided above can strengthen the evidence however; the critical citations have been included already.

Response: following reviewer's suggestion, considering above added text/references in '[Overview for Molecular Initiating Event](#)' (in Event 424: Inhibition, Na⁺/I⁻ symporter (NIS)), we changed Life Stage Applicability into 'high'.

Regulatory Applicability

Since the AOP covers endpoints that are measured using widely accepted methods, including TH levels and memory, learning and IQ, it is highly probable that it will have broad regulatory applicability. This AOP can provide the basis for standardizing evaluation of classes of chemicals and their biological impact. The weight of evidence and classifications of the KEs and KERs provides an important framework to guide policy/regulatory development.

Conclusion

The AOP is very well developed. The revisions suggested provide possible enhancements to the AOP but the central tenets are strong and well supported.

Reviewer#3

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?

1. The broad hypothesis of AOP 54 is based on the strong scientific evidence that thyroid hormone is critical for normal neurodevelopment, such that the MIE of sodium iodide symporter (NIS) inhibition results in several KEs corresponding to decreased thyroid hormone availability and synaptogenesis defects to result in the AO of decreased learning and memory. This AOP is well-written and presented with good biological plausibility.

However, the specified KEs represent one potential pathway toward the AO. Brain development is complex, and there are likely other KEs and AOs which can follow the MIE.

Response: If there are data to support the assumption that the same MIE can trigger the cascade of different KEs at different biological levels leading to various AOs at organism level, the new AOPs should be developed. These AOPs could be connected into AOPs network to illustrate toxicity pathways triggered by disruption of TH homeostasis, resulting in various AOs.

c. KEs related to decreased neuronal thyroid hormone levels: Thyroid hormone-dependent actions in the brain not only include synaptogenesis, but also neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone projections.

Response: Indeed, TH plays an important role in brain development as we have described in the **Overall Assessment of the AOP** (see text below)

"Neonatal hypothyroidism results in altered neuronal structure and function, including reduction in neurite outgrowth, synaptogenesis and dendritic elaborations. RC3/neurogranin is a gene directly regulated by thyroid hormone whose expression is consistent with a role in synapse formation and/or function (Munoz et al., 1991). The specific alterations in dendritic morphology have been identified in several cell types, including pyramidal cells in the cerebral cortex (decrease in dendritic spine number) (Schwartz, 1983), pyramidal cells in the visual cortex (reduced number and altered distribution of

dendritic spines) (Morreale de Escobar et al., 1983), cholinergic basal forebrain neurons (decreased number of primary dendrites and number of dendritic branch points) (Gould and Butcher, 1989), Purkinje cells (decreased number and size of dendritic spines) (Nicholson and Altman, 1972; Legrand, 1979) and granule and pyramidal cells in the hippocampus (decreased branching of apical and basal dendrites) (Rami et al., 1986). Thus, TH influences the size, packing density and dendritic morphology of neurons throughout the brain, including myelination. Indeed, a striking phenotype in the hypothyroid neonatal brain is the reduction in myelin-protein gene expression (Farsetti et al., 1992; Pombo et al., 1999)".

As already described, synaptogenesis is the fundamental unit of connectivity and communication between neurons, playing a vital role in synaptic plasticity, learning and memory and adaptation throughout life. It follows early neurodevelopmental processes such as neuronal and glial cells proliferation, migration, alterations in dendritic arborisation etc., therefore it encompasses, possible changes in these early stages of brain development that could also be triggered under hypothyroidism, leading to defective synaptogenesis and resulting in abnormal function of neuronal network function, the last two KEs that lead to AO, defined here as impairment of learning and memory. This has been briefly discussed under KER 358: Synaptogenesis, Decreased leads to Neuronal Network Function decreased

Changes in synaptogenesis are directly linked to the KE downstream Neuronal network Function which can be evaluated by measuring neuronal electrical activity (spontaneous or evoked). It is a neuronal specific, functional assay linked to synaptogenesis that should be much more reliable than measurements of gene or protein expression alterations.

These two KEs 'Decreased Synaptogenesis' and 'Decreased Neuronal function' are also defined as two last KEs leading to AO (Impairment of learning and memory) in other two AOPs (13 and 48) which are already endorsed by WNT.

If there are evidence that changes in other neurodevelopmental processes such as neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone projections could also be defined as KEs leading to other AOs, than hopefully new AOPs will be developed.

2. The preponderance of these other KEs would be based on primarily animal data, with the exception of hippocampal defects that does have both animal and human data. It would be worth considering to include the hippocampal work of Drs. Mary Gilbert, Joanne Rovet, and colleagues as KEs.

Response: We have cited M. Gilbert's work through the whole AOP description where relevant, referring to the below published papers:

Gilbert ME. (2004). Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. Brain Res Dev Brain Res 148:11-18.

Gilbert ME. (2011). Impact of low-level thyroid hormone disruption induced by propylthiouracil on brain development and function. Toxicol Sci 124:432-445.

Gilbert ME, Hedge JM, Valentin-Blasini L, Blount BC, Kannan K, Tietge J, Zoeller RT, Crofton KM, Jarrett JM, Fisher JW. (2013). An animal model of marginal iodine deficiency during development: the thyroid axis and neurodevelopmental outcome. *Toxicol Sci* 132:177-195.

Gilbert ME, Lasley SM. (2013). Developmental thyroid hormone insufficiency and brain development: a role for brain-derived neurotrophic factor (BDNF)? *Neurosci* 239: 253-270.

Gilbert ME, Paczkowski C. (2003). Propylthiouracil (PTU)-induced hypothyroidism in the developing rat impairs synaptic transmission and plasticity in the dentate gyrus of the adult hippocampus. *Brain Res Dev Brain Res* 145:19-29.

Gilbert ME, Sanchez-Huerta K, Wood C. (2016). Mild Thyroid Hormone Insufficiency During Development Compromises Activity-Dependent Neuroplasticity in the Hippocampus of Adult Male Rats. *Endocrinology* 157:774-787.

Gilbert ME, Sui L. (2006). Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* 1069:10–22.

Gilbert ME, Sui L. (2008). Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat. *Environ Health Perspect* 116:752–760.

Gilbert ME, Sui L, Walker MJ, Anderson W, Thomas S, Smoller SN, Schon JP, Phani S, Goodman JH. (2007). Thyroid hormone insufficiency during brain development reduces parvalbumin immunoreactivity and inhibitory function in the hippocampus. *Endocrinology* 148:92–102.

As suggested, the additional, following papers are now discussed and cited in the relevant KERs:

Gilbert ME, Rovet J, Chen Z, Koibuchi N. (2012). Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences. *Neurotoxicology*. 33(4):842-52. (in Overall AOP assessment).

Gilbert ME, Hedge J, Grant K, Lyke D, Gitata I, Anderson W, et al. Marginal iodide deficiency, thyroid hormones, and neurodevelopment: developing a model. *Toxicol Sci* 2009;108 (S-1):32 (in KER 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased).

Gilbert M, Hedge J, Zoeller RT, Kannan K, Crofton K, Valentin-Blasini L, et al. Developmental iodide deficiency: reductions in thyroid hormones and impaired hippocampal transmission. *Toxicol Sci* 2011;120(S-2):558 (in KER 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased).

Gilbert ME. Impact of low-level thyroid hormone disruption induced by propylthiouracil on brain development and function. *Toxicol Sci* 2011;124:432–45 (in indirect KER: decreased TH synthesis and AO)

Gilbert ME, McLanahan ED, Hedge J, Crofton KM, Fisher JW, Valentin-Blasini L, Blount BC. (2011). Marginal iodide deficiency and thyroid function: dose-response analysis for quantitative pharmacokinetic modeling. *Toxicology*. 283(1):41-8. (in KER 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased).

This below text (as already commented in reply to Reviewer #1) has been inserted into: KER 872 'Iodine deficiency leads to decrease TH synthesis' in **Quantitative Understanding of the Linkage**:

- Gilbert et al., 2011: This study examined the relationship between graded levels of iodine (ID) in rats and serum thyroid hormones, thyroid iodine content, and urinary iodide excretion. The study provided

parametric and dose-response information for development of a quantitative model of the thyroid axis. Female Long Evans rats were fed casein-based diets containing varying iodine (I) concentrations for 8 weeks. Diets were created by adding 975, 200, 125, 25, or 0 µg/kg I to the base diet (~25 µg I/kg chow) to produce 5 nominal I levels, ranging from excess (basal+added I, Treatment 1: 1000 µg I/kg chow) to deficient (Treatment 5: 25 µg I/kg chow). Food intake and body weight were monitored throughout and on 2 consecutive days each week over the 8-week exposure period, animals were placed in metabolism cages to capture urine. Food, water intake, and body weight gain did not differ among treatment groups. Serum T4 was dose-dependently reduced relative to Treatment 1 with significant declines (19 and 48%) at the two lowest I groups, and no significant changes in serum T3 or TSH were detected. Increases in thyroid weight and decreases in thyroidal and urinary iodide content were observed as a function of decreasing ID in the diet. Data were compared with predictions from a published biologically based dose-response (BBDR) model for ID. These results challenged existing models and provide essential information for development of quantitative BBDR models for ID during pregnancy and lactation.

Notably, the strength of evidence underlying the relationship between low thyroid hormone levels and hippocampal deficits is likely stronger than that between low thyroid hormone levels and decreased BDNF (regarded as low strength of evidence by the AOP authors and as written about on page 54: BDNF is thought to underlie the effects of developmental hypothyroidism but this notion is based mainly on their common physiological role during brain development rather than on solid experimental evidence [Gilbert and Lasley, 2013].)

Response: As pointed out by Reviewer #4, new semi quantitative studies have been now cited (Shafiee et al., 2016) in support of this KER 444 (T4 in neuronal tissue, Decreased leads to BDNF, Reduced). In addition, in reply to Reviewer #1, other data are now discussed in support to this KER 444, including the most recently published reports with some quantitative data (please, see pages 11-14, describing the following studies: Sui et al. 2010; Blanco et al. 2013; Abedelhaffez and Hassan, 2013; Shafiee et al., 2016; Shi et al. 2017; Mokhtari et al. 2017; Sabbaghziarani et al. 2017; Kawahori et al. 2018).

Could the Reviewer #3 be more specific and explain what he/she means by hippocampal deficits, how to measure it and how to link it to KE downstream (KER).

d. AOs: In addition to deficits in learning and memory, decreased IQ in children has specifically been studied quite extensively as a result of maternal hypothyroidism during pregnancy. Both the current AO and decreased IQ are more commonly grouped under the global term of decreased cognition, and thus this could also be added as a critical AO.

Response: IQ measurements are also tools to measure learning and memory deficits. We defined AO in a more specific manner "learning and memory impairment" since this endpoint evaluation is required by the current OECD TG DNT 426. It is also defined as AO in other five AOPs, and three of them (12, 13, and 48) have been already endorsed by WNT.

It is important that a variety of tests, specific for different types of learning and memory exists in laboratory animals and humans as described under Adverse Outcome: How It Is Measured or Detected. Hopefully, in the future, this AOP can contribute to development of testing strategy (such as IATA) that will permit to identify chemicals with potential to cause impairment of learning and memory, leading to refinement (or possibly replacement, in the future) of animal testing.

2. 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 dysidenin and aryltrifluoroborates as stressors to the MIE are not particularly strong, and probably low to moderate at best. There are multiple other stressors which can inhibit NIS that have greater applicability, thus these two are not those which one usually associates with this MIE.

Response: As suggested, we changed the stressors strength evaluation for **dysidenin and aryltrifluoroborates as low/moderate.**

Please, let us know which other chemicals (besides perchlorate, thiocyanate and nitrate) could be considered: *"There are multiple other stressors which can inhibit NIS that have greater applicability"*

Regarding the life stage applicability of the MIE specifically, there are significant data supporting the concept that decreased thyroid hormone resulting from NIS inhibition is most relevant during early life when neurodevelopment begins, thus I would recommend increasing the strength of evidence during the three listed life stages from moderate to strong.

Response: we changed it from moderate to strong, as suggested.

Otherwise, I agree with the weight of evidence designations assigned to each of the KEs, KERs, and the overall AOP.

3. Regulatory applicability

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

The point presented in the Considerations for Potential Applications of this AOP would be supported by the evidence presented. As Integrated Approaches and Testing Assessment (IATA) strategies take into account an acceptable level of uncertainty and not all of the intermediate KEs need to be quantified, this AOP would provide an initial basis for the identification of substances to identify chemicals acting through this pathway.

One important consideration that is not noted, however, is that the potential severity of the AO can be mitigated by adequate iodine nutrition to overcome the effects of the MIE; the mention of iodine status is absent in this section and is crucial toward potential regulatory applicability of this AOP.

Response: As suggested, the following text (see below) on severity of the AO that can be mitigated by adequate iodine nutrition has been inserted in the section on KER 872 description (Thyroidal Iodide, Decreased leads to TH synthesis, Decreased):

"Concern about environmental perchlorate exposure is focused on its inhibition of iodide uptake into the thyroid (MIE). Decreased iodine intake may decrease thyroid hormone production. Perchlorate exposure, therefore, might be particularly detrimental in iodine-deficient individuals. Median urinary iodine levels are used instead and reflect dietary iodine sufficiency across populations (International Council for the Control of Iodine Deficiency Disorders (ICCIDD); available from: www.iccidd.org). According to

ICCIDD report Iodine deficiency continues to be an important global public health issue, with an estimated 2.2 million people (38% of the world's population) living in iodine-deficient areas. In 1990, the United Nations World Summit for Children set forth the goal of eliminating iodine deficiency worldwide (UNICEF World Summit for Children. Available from: <http://www.unicef.org/wsc/declare.htm>; 1990). Considerable progress has been achieved by programmes of universal salt iodisation (USI) in various countries, in line with the recommendations of the World Health Organization (WHO) (WHO, UNICEF, ICCIDD. A guide for programme managers. World Health Organization; Geneva: 2007. Assessment of the iodine deficiency disorders and monitoring their elimination.WHO/NHD/01.1). However, many countries remain iodine deficient (de Benoist et al., 2013; Lazarus and Delange, 2004). In the U.S., data from large population studies have shown that median urinary iodine levels decreased by approximately 50% between the early 1970s and the early 1990s, although the population overall remained iodine sufficient (Hollowell et al., 1998). Subsequent studies have shown that this decrease has stabilised (Caldwell et al., 2005).

The WHO still considers iodine deficiency, which leads to hypothyroidism, the single most important preventable cause of brain damage worldwide (WHO/UNICEF/ICCIDD, 2007). The most vulnerable groups are pregnant and lactating women and their developing fetuses and neonates, given the crucial importance of iodine to ensure adequate levels of thyroid hormones for brain maturation. Iodine deficiency in pregnancy is a prevailing problem not only in developing countries, but also in western industrialized nations and other countries classified as free of iodine deficiency, and solution may be found in dietary changes (Moog et al., 2017).

References (inserted in KER 872):

de Benoist B, Andersson M, Takkouche B, et al. Prevalence of iodine deficiency worldwide. *Lancet*. 2003; 362:1859–1860.

Lazarus JH, Delange F. Prevalence of iodine deficiency worldwide. *Lancet*. 2004; 363:901.

Hollowell JG, Staehling NW, Hannon WH, et al. Iodine nutrition in the United States. Trend and public health implications: iodine excretion data from National Health and Nutrition Examination Survey I and III (1971-1974 and 1988-1994). *The Journal of Clinical Endocrinology and Metabolism*. 1998; 83:3401–3408.

Caldwell KL, Jones R, Hollowell JG. Urinary iodine concentration: United States National Health and Nutrition Examination Survey 2001-2002. *Thyroid*. 2005; 15:692–699.

Moog N.K., Entringer S., Heim Ch., Wadhwa PD., Kathmann N., Buss C.: Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience*, 2017, 342: 68–100.

4. Conclusion

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

Broadly, AOP 54 is well-prepared and has been comprehensively organized. There are some concerns regarding the strength of evidence underlying the KEs and KERs, and there are certainly others which may offer a higher level of evidence linking the MIE with the AO. In addition, the AO is fairly specific, and other neurodevelopment defects well-known to result from decreased thyroid hormone levels are not represented.

Response: could the reviewer explain what "*other neurodevelopmental defects well-known to result from decreased thyroid hormone levels*" could be defined as additional AO(s) at the organism level, triggered by NIS inhibitors and mediated through the same sequence of key events. If such AOs (besides learning and memory impairment) exist, we will consider them.

Finally, regulatory applicability of the AOP must take into account iodine status and supplementation, and further emphasis on this important mediator can be made in the AOP. Overall, the authors have developed a thoughtful summary of the current concepts underlying this pathway.

Response: As mentioned above, the text on iodine supplementation has been inserted in the description of KER 872.

Reviewer#4

AOP external review – 2018 – AOP 54: Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment.

General comments

The AOP is very well described and of good quality and the available studies are adequately judged.

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?

h) For MIE, Inhibition of Na⁺/I⁻ symporter (NIS): please refer to the OECD new scoping document and the references used within this document in the paragraph “how to measured or detected”. Title document: OECD Series on Testing and Assessment (2017) New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling” the NIS assay at page 36-38.

Response: We know the OECD scoping document very well but we have not cited it since we referred to specific papers, describing NIS inhibition assays (included in the OECD scoping document). As suggested we also cited the OECD document.

i) KE ‘T4 in serum, decreased’ (page 8-11): paragraph “how it is measured or detected”

d) Please specify what the advantages and disadvantages are for measuring free and total T4 and T3 and what the preference has to measure this KE.

Response: According to the Guidelines on Newborn Screening and Therapy for Congenital Hypothyroidism (Smith, 2007; Smith L., Am Fam Physician. 2007 Aug 1;76(3):439-444; Practice Guidelines: Updated AAP Guidelines on Newborn Screening and Therapy for Congenital Hypothyroidism) the screening strategies for the detection of congenital hypothyroidism are based on the measurement of T4 and TSH levels and it should be performed for all infants between two and four days of birth.

However, taking into consideration that notably T3 exerts its actions by binding to nuclear TH receptors, which regulate transcription of target genes, measurement of T3 is also important. Measuring only T4 is not reliable enough, as even high level of T4 in the case of D2 dysfunction, could result in low levels of T3.

e) Different techniques are mentioned to measure thyroid blood levels. Two are missing namely HPLC-MS and Immuno Luminescence. All the available assays have different sensitivities. Therefore, results including reproducibility and repeatability really depends on the protocol used. Standardization of

analysis for this KE is crucial to make comparisons between independent experiments possible and to better judge the effects in TH levels (Chang et al., 2007).

Response: In the text we have described [radioimmunoassay](#) and HLPC and mass spectrometry methods with the relevant references:

"Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is [radioimmunoassay \(RIA\)](#). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is a commonly used as a human clinical test method. Least common is [analytical determination of iodothyronines \(T3, T4, rT3, T2\) and their conjugates, though methods employing HLPC and mass spectrometry exist \(Hornung et al., 2015; DeVito et al., 1999; Spencer, 2013\).](#)"

As suggested, missing immune-luminescence assay has been inserted:

"T4 can also be measured using immunoluminescence assay (Baret and Fert, 1989). The specific amplification (the xanthine oxidase dependent luminescence of luminol enhanced in the presence of Fe-EDTA complex) has been applied to T4 immunoassays, with T4-XO (xanthine oxidase). The performances of these assay is at least equivalent to those obtained with iodinated tracers, using the same solid phases and the same calibrators. The major advantages of these immunoassays are: (1) the long-term signal which can be repeatedly recorded over several days, (2) the high detection sensitivity, (3) the long-term stability of the luminescence reagent, and (4) the stability of the conjugates."

Reference (inserted in KE 281: Thyroxine (T4) in serum, Decreased)

Baret A. and Fert V. (1989) T₄ and ultrasensitive TSH immunoassays using luminescent enhanced xanthine oxidase assay. *Luminescence, the journal of Biological and Chemical Luminescence*. 4(1):149-153.

f) Please mention that blood sampling should be controlled for experimental factors (such as circadian rhythm or food intake) that might influence the measured concentration measured and the variability of the hormone determination (Döhler et al., 1979).

Response: According to our TC discussion these factors should not be mentioned in the AOP text. However, we leave the final decision to our Reviewers.

This below text can be inserted if still required (under KE 281: Thyroxine (T4) in serum, Decreased):

"If rat model is used as experimental animal for measurements of T4 and T3 levels in blood samples the following factors should be taken into consideration: sex basal serum levels of T4 and T3, changes during different age stages development of male and female rats, environmental temperature, housing, animal handling (level of stress), methods of blood collection. Distinct cardiac changes have been reported to be also linked to serum TSH levels, but the context of T4 or T3 are not discussed (Döhler et al., 1979)."

Reference (inserted in KE 281: Thyroxine (T4) in serum, Decreased)

Döhler Wong CC, von zur Mühlen A (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. *Pharmac Ther* 5: 305-318.

j) For KE “T4 in neuronal tissue, decreased”, paragraph ‘how it is measured and detected’ (page 11-13). Based on the brain region specific levels it is important not to measure whole brain levels but also brain region specific TH levels although that might be quite hard (Constantinou et al., 2005).

Response: The following text has been inserted into [KE 280 description: Decrease of T4 in neuronal tissue](#):

"The data published by Constantinou et al., (2005) determined whether changes in the circulating thyroid hormone (TH) and brain synaptosomal TH content affected the relative levels of mRNA encoding different thyroid hormone receptor (TR) isoforms in adult rat brain. Region-specific quantitative differences in the expression pattern of all thyroid hormone receptor isoforms in euthyroid animals and hypothyroid animals were recorded and the obtained results show that *in vivo* depletion of TH regulates TR gene expression in adult rat brain in a region-specific manner (Constantinou et al., 2005)."

Reference (inserted in KE 280):

Constantinou C, Margarity M, Valcana T. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. *Molecular and Cellular Biochemistry*. 2005, 278 (1–2): 93–100.

Please mention that the way of dissecting the brain regions is crucial to draw the right conclusions. It is also possible to measure other aspects of the thyroid functioning since reporters and enzymes affect levels in specific brain regions (Moog et al., 2017).

Response: As suggested, the following text has been inserted (under [KE 280 description: Decrease of T4 in neuronal tissue](#)):

"Distribution of TH in the brain differs between brain regions; for instance midbrain and telencephalon appear to be places of high signalling. This signalling pattern in adult mouse is well correlated with T3 distribution measured in various brain areas (Constantinou et al., 2005; Chatonnet et al., 2011; Moog et al., 2017).

Reference (inserted in KE 280):

Constantinou C, Margarity M, Valcana T. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. *Molecular and Cellular Biochemistry*. 2005, 278 (1–2): 93–100.

Chatonnet F., Picou F., Fauquier T., and Flamant F., (2011) Thyroid Hormone Action in Cerebellum and Cerebral Cortex Development, *Journal of Thyroid Research*, Volume 2011, Article ID 145762, 8 pages <http://dx.doi.org/10.4061/2011/145762>)

Moog N.K., Entringer S., Heim Ch., Wadhwa PD., Kathmann N., Buss C.: Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience*, 2017, 342: 68–100.

k) For KE “Reduced levels of BDNF”, please focus on a brain region or mentioned the “most likely affected brain regions” such as the hippocampus and cortex and include references (Koromilas et al., 2010, Shafiee et al., 2016).

Response: As suggested, in the description of KER 444 (T4 in neuronal tissue, Decreased leads to BDNF) (not in KE, as only KER describes relationship between TH and BDNF levels) the following text has been inserted:

"It has been shown that the thyroid insufficiency (lower TH levels) results in reduction of BDNF levels (mRNA or protein) in the developing brain, and the most likely affected brain regions are the hippocampus and cortex (Koromilas et al., 2010, Shafiee et al., 2016). The hippocampus direct and indirect interactions with the THs provide crucial information on the neurobiological basis of the hypothyroidism-induced mental retardation and neurobehavioral dysfunction. TH deficiency during the foetal and/or the neonatal period produces deleterious effects for neural growth and development (such as reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, deranged axonal projections, decreased synaptogenesis and alterations in neurotransmitters' levels), possibly through decreased BDNF levels (Koromilas et al., 2010; Shafiee et al., 2016)."

References (inserted in KER 444):

Koromilas CI, Liapi C, Schulpis KH, Kalafatakis K, Zarros A, Tsakiris S. Structural and functional alterations in the hippocampus due to hypothyroidism. *Metab Brain Dis.* 2010 Sep;25(3):339-54.

This paper by Shafie et al., (2016) has been already cited in the original text of KER 444.

Shafiee SM, Vafaei AA, Rashidy-Pour A. Effects of maternal hypothyroidism during pregnancy on learning, memory and hippocampal BDNF in rat pups: Beneficial effects of exercise. *Neuroscience.* 2016 Aug 4;329:151-61.

1) Nothing is mentioned in the KEs about the effects of maternal hypothyroidism. It is likely that during prenatal brain development effects of disruption of the thyroid levels might be more severe than during adulthood, although effects of hypothyroidism on neuronal functioning occurs throughout life (Moog et al., 2017, Préau et al., 2015)

Response: This AOP is focused on NIS inhibition as MIE and it refers to both foetus or mother inhibited TH synthesis, which will finally result in hypothyroidism. We added some text to point out also contribution to AO of maternal hypothyroidism under KER 442 (Na⁺/I symporter inhibition leads to decreased thyroidal iodine):

"The effects of maternal hypothyroidism could also contribute to this KER. During pregnancy TH requirements increase, particularly during the first trimester (Alexander et al. 2004; Leung et al. 2010), due to higher concentrations of thyroxine-binding globulin, placental T4 inner-ring deiodination leading to the inactive reverse T3 (rT3), and transfer of small amounts of T4 to the foetus (during the first trimester foetal thyroid function is absent). Moreover, glomerular filtration rate and clearance of proteins and other molecules are both increased during pregnancy, possibly causing increased renal iodide clearance and a decreased of circulating plasma iodine (Glinoe, 1997). Thus, even though the foetal thyroid can trap iodide by about 12 week of gestation (Fisher and Klein, 1981), high concentrations of maternal perchlorate may potentially decrease thyroidal iodine available to the foetus by inhibiting placental NIS (Leung et al. 2010)."

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show visual attention, visual processing and gross motor skills deficits, while if it occurs later, offspring may show subnormal visual and visuospatial skills, along with slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004).

There are limited data regarding low-level environmental perchlorate exposure and maternal thyroid function during pregnancy. A Chilean study found no increases in TSH or decreases in free thyroxine or urinary iodine concentrations in pregnant women living in three areas (all of which had more than adequate mean urinary iodine levels) with long-term environmental perchlorate exposure (Télez Télez et al. 2005). A follow-up analysis of this cohort also confirmed the lack of association between individual urinary iodide or perchlorate concentrations and thyroid function in the pregnant women (Gibbs and Van Landingham, 2008). Studies of large cohorts of first-trimester pregnant women from the U.S., Europe and Argentina found that environmental perchlorate exposure did not affect maternal thyroid function (Pearce et al. 2009).

In 'Uncertainties and Inconsistencies' (KER 442) the following text has been inserted: "The data assessing the effect of maternal perchlorate exposure in neonates and children and thyroid function remain unclear (Leung et al., 2010)."

Reference (inserted in KER 442):

Alexander EK, Marqusee E, Lawrence J, Jarolim P, Fischer GA, Larsen PR (2004). Timing and magnitude of increases in levothyroxine requirements during pregnancy in women with hypothyroidism. *N Engl J Med.* 2004 Jul 15;351(3):241-9.

Fisher DA, Klein AH (1981). Thyroid development and disorders of thyroid function in the newborn. *N Engl J Med.* 1981 Mar 19;304(12):702-12.

Gibbs JP, Van Landingham C (2008). Urinary perchlorate excretion does not predict thyroid function among pregnant women. *Thyroid.* Jul; 18(7):807-8.

Glinoe D (1997). The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev.* Jun;18(3):404-33.

Leung AM, Pearce EN, Braverman LE. Perchlorate, iodine and the thyroid. Best Pract Res Clin Endocrinol Metab. 2010 Feb;24(1):133-41.

Pearce EN, Lazarus JH, Smythe PP, et al. (2009). Thyroid Function is Not Affected by Environmental Perchlorate Exposure in First Trimester Pregnant Women. *Endocrine Society 91st Annual Meeting; USA.*

Télez Télez R, Michaud Chacón P, Reyes Abarca C, Blount BC, Van Landingham CB, Crump KS, Gibbs JP (2005). Long-term environmental exposure to perchlorate through drinking water and thyroid function during pregnancy and the neonatal period. *Thyroid.* Sep; 15(9):963-75.

Zoeller RT, Rovet J. (2004). Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. *J Neuroendocrinol.*,16(10):809-18.

m) From a neuroscientific point of view, the models measuring the KE are very generally written at this stage. In future neuroscientific expertise on how the KE can be measured properly might help to make the best choice for a model to study the Key event.

Response: For the quantitative AOP evaluation it is less important to measure a KE on its own. The most important is to measure the changes in KE upstream simultaneously with the changes in relevant KE

downstream. In such, it will be possible to determine to what extent KE up stream has to be disturbed by an exposure to a chemical to trigger the changes in the relevant KE downstream.

At this stage the general description of the methods for measuring KEs is advisable, following the OECD guidance (OECD (2018), "Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways", OECD Series on Adverse Outcome Pathways, No. 1, OECD Publishing, Paris. <http://dx.doi.org/10.1787/5jlv1m9d1g32-en>): *"One of the primary considerations in evaluating AOPs is the relevance and reliability of the methods with which the KEs can be measured. The aim of this section of the KE description is not to provide detailed protocols, but rather to capture, in a sentence or two, per method, the type(s) of measurements that can be employed to evaluate the KE and the relative level of scientific confidence in those measurements"*.

n) Include references at page 20 by the sentence: "patch clamping technique can also be used to measure neuronal network activity" (e.g. Bosca et al., 2014).

Response: As suggested, the following sentence and the relevant reference have been inserted (in [KE 386: Decrease of neuronal network function](#)):

"In some cases, if required, planar patch clamping technique can also be used to measure neuronal networks activity (e.g., Bosca et al., 2014)."

Reference:

Bosca, A., M. Martina, and C. Py, Planar patch clamp for neuronal networks--considerations and future perspectives. *Methods Mol Biol*, 2014. 1183: p. 93-113.

o) Thyroid levels can also affect synaptogenesis via other pathways (not only BDNF). The authors can consider including an indirect KER between T4 serum levels and synaptogenesis (Robichaux et al., 2014)?

Response: In this AOP we proposed to focus on the role of BDNF in the context of synaptogenesis, as dysfunction of BDNF is strongly involved in neuronal differentiation and maturation, which plays a role in pathophysiology of learning and memory processes described in the indirect KER 1507 (Reduced BDNF and Impairment of learning and memory).

In the paper by Robichaux et al., 2014 different pathways that regulate synaptogenesis are described; however, in this paper it is not discussed which pathways involved in synaptogenesis are under the control of TH signalling.

If there is evidence for other pathways involved in the regulation of synaptogenesis triggered by reduced TH, another AOP(s) should be developed and linked through the AOPs network with this one.

2. 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 judgement by the AOP developers for the KER is very clearly and accurately described, the available studies are very well judged and all the uncertainties are described accurately.

3. 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 developmental (neuro) toxicity and for identification of endocrine disruptors (thyroid disruptors). Additionally, this AOP can be used and help to unravel the mechanisms of thyroid hormone disruption and the occurrence of learning and memory impairment. Therefore, it is probable that it will be applicable for mechanistic tests as part of an IATA. The AOP is very interesting since it describes an important aspect of thyroid disruption for which many epidemiological evidence is available.

4. Conclusion:

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

I would recommend this AOP for submission. An AOP is intended to be a constantly developing document, the adverse outcome is very important and proven to occur after hypothyroidism. It nicely links epidemiological evidence with mechanistic data. A more specific description of available models to measure the KE is needed in future. Specific expertise on the models from neuroscientist would be helpful, since these KE are difficult to measure. The AOP is very well described and of good quality.

As suggested by Reviewer #4, all below references have been cited in the relevant AOP text:

References:

Bosca, A., M. Martina, and C. Py, Planar patch clamp for neuronal networks--considerations and future perspectives. *Methods Mol Biol*, 2014. 1183: p. 93-113.

Chang SC1, Thibodeaux JR, Eastvold ML, Ehresman DJ, Bjork JA, Froehlich JW, Lau CS, Singh RJ, Wallace KB, Butenhoff JL. Negative bias from analog methods used in the analysis of free thyroxine in rat serum containing perfluorooctanesulfonate (PFOS). *Toxicology*. 2007 May 5;234(1-2):21-33.

Constantinou C, Margarity M, Valcana T. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. *Molecular and Cellular Biochemistry*. October 2005, Volume 278, Issue 1-2, pp 93-100.

Döhler et al. (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. *Pharmac Ther* 5: 305-318.

Koromilas C, Liapi C, Schulpis KH, Kalafatakis K, Zarros A, Tsakiris S. Structural and functional alterations in the hippocampus due to hypothyroidism. *Metab Brain Dis*. 2010 Sep;25(3):339-54.

Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N, Buss C. Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience*. 2017 Feb 7;342:68-100.

Robichaux MA, Cowan CW. Signaling mechanisms of axon guidance and early synaptogenesis. *Curr Top Behav Neurosci*. 2014 (*not inserted as it is not relevant in the context of TH*)

Shafiee SM, Vafaei AA, Rashidy-Pour A. Effects of maternal hypothyroidism during pregnancy on learning, memory and hippocampal BDNF in rat pups: Beneficial effects of exercise. *Neuroscience*. 2016 Aug 4;329:151-61.

OECD no. 207: New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. <http://www.oecd-ilibrary.org/docserver/download/9717091e.pdf?expires=1517915713&id=id&accname=guest&checksum=B30ECD7FE792AD539750CCE7AC8B15E5>.

Additional modifications introduced based on TC discussion (18-05-2018):

We introduced in MIE description (Inhibition, Na⁺/I⁻ symporter) data of two studies suggested by Kevin Crofton:

Hallinger DR, Murr AS, Buckalew AR, Simmons SO, Stoker TE, Laws SC (2017). Development of a screening approach to detect thyroid disrupting chemicals that inhibit the human sodium iodide symporter (NIS). *Toxicol In Vitro*. Apr;40:66-78.

Wang J, Hallinger DR, Murr AS, Buckalew AR, Simmons SO, Laws SC, Stoker TE (2018). High-Throughput Screening and Quantitative Chemical Ranking for Sodium-Iodide Symporter Inhibitors in ToxCast Phase I Chemical Library. *Environ Sci Technol*. 2018 May 1;52(9):5417-5426.

We added the following text:

"The U.S. EPA's Endocrine Disruptor Screening Program aims to use high-throughput assays and computational toxicology models to screen and prioritize chemicals that may disrupt the thyroid signaling pathway. Thyroid hormone biosynthesis requires active iodide uptake mediated by the sodium/iodide symporter (NIS). Monovalent anions, such as the environmental contaminant perchlorate, are competitive inhibitors of NIS, yet limited information exists for more structurally diverse chemicals. A novel cell line expressing human NIS, hNIS-HEK293TEPA, was used in a radioactive iodide uptake (RAIU) assay to identify inhibitors of NIS-mediated iodide uptake. The RAIU assay was optimized and performance evaluated with 12 reference chemicals comprising known NIS inhibitors and inactive compounds. An additional 39 chemicals including environmental contaminants were evaluated, with 28 inhibiting RAIU over 20% of that observed for solvent controls. Cell viability assays were performed to assess any confounding effects of cytotoxicity. RAIU and cytotoxic responses were used to calculate selectivity scores to group chemicals based on their potential to affect NIS. RAIU IC₅₀ values were also determined for chemicals that displayed concentration-dependent inhibition of RAIU (≥50%) without cytotoxicity. Strong assay performance and highly reproducible results support the utilization of this approach to screen large chemical libraries for inhibitors of NIS-mediated iodide uptake (Hallinger et al., 2017).

This study (Wang et al., 2018) applied a previously validated high-throughput approach to screen for NIS inhibitors in the ToxCast phase I library, representing 293 important environmental chemicals. Here 310 blinded samples were screened in a tiered-approach using an initial single-concentration (100 μM) radioactive-iodide uptake (RAIU) assay, followed by 169 samples further evaluated in multi-

concentration (0.001 µM–100 µM) testing in parallel RAIU and cell viability assays. A novel chemical ranking system that incorporates multi-concentration RAIU and cytotoxicity responses was also developed as a standardized method for chemical prioritization in current and future screenings. Representative chemical responses and thyroid effects of high-ranking chemicals are further discussed. This study significantly expands current knowledge of NIS inhibition potential in environmental chemicals and provides critical support to U.S. EPA's Endocrine Disruptor Screening Program (EDSP) initiative to expand coverage of thyroid molecular targets, as well as the development of thyroid adverse outcome pathways (AOPs)."

Based on the TC conclusion we changed the *Weight of Evidence* from 'weak' to 'moderate' in KER 444 (T4 in neuronal tissue, Decreased leads to BDNF, Reduced).

In KE 444: We added the following comment on the technical issue of performing BDNF measurement in the brain tissue:

"Measuring BDNF levels changes in the brain, especially when low, at the boarder to be significant are technically difficult."

We also mentioned that reduced TH levels may trigger several other effects, apart from synaptogenesis decrease, as already described in response to Review #4 (see KER 444 (T4 in neuronal tissue, Decreased leads to BDNF Reduced), where the following text has been inserted: "TH deficiency during the foetal and/or the neonatal period produces deleterious effects for neural growth and development (such as reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, deranged axonal projections, decreased synaptogenesis and alterations in neurotransmitters' levels), possibly through decreased BDNF levels (Koromilas et al., 2010; Shafiee et al., 2016)."

Similar comment was added also under KER 448: BDNF, Reduced leads to Synaptogenesis, Decreased (under 'Biological plausibility'):

"TH deficiency during the foetal and/or the neonatal period, apart from reducing synaptogenesis, can produce several other deleterious effects for neural growth and development (e.g., such as reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, deranged axonal projections, and alterations in neurotransmitters' levels), possibly through decreased BDNF levels (Koromilas et al., 2010; Shafiee et al., 2016)." We added (Koromilas et al., 2010) study to the reference list in this KER.

These studies are relevant to both KERs (444 and 448) therefore, are cited twice.

Under same KER 448 (in 'Key Event Relationship Description'), we added a comment on other relevant factors, apart from BDNF, involved in synaptogenesis regulation under TH signaling (e.g., NGF, Reelin, etc.).

We made sure that in the text throughout the AOP it is clearly stated which type of studies, in vivo, in vitro or epidemiological were described, if not already specified.

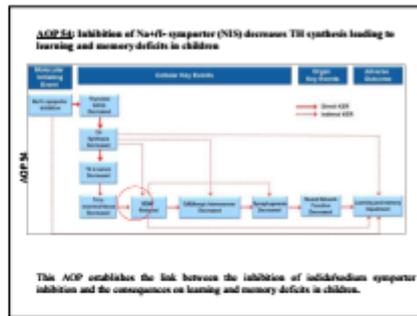
We commented on TH role during brain development, as a complex and still not fully understood process (in Overall AOP evaluation).

Evaluation of the strength of the common KERs between our AOP 54 and AOP 42 has been aligned.

MIE: NIS inhibition

- Other stressors than perchlorate, thiocyanates or nitrates are cited as "strong" however dysidenin and aryl-trifluoroborates are not particularly strong but more probably low to moderate **_ will be done**
- Other parameters could influence the effects of a given chemical stressor. Such parameters as age, gender, pregnancy as well as thyroid status and dietary levels should be described in greater detail in the AOP **_ will be included**
- Recent available data in scientific literature indicate that exposure to perchlorate (among other compounds) during pregnancy is detrimental.

Major discussion on evidence about perchlorate leading to cognitive defects needs discussion. **_ new data incorporated**



KE 381 and KER 444

Before revision reviewers indicated that BDNF levels reduction needed refinement.

- Questions about the quantification of dose dependent relationship between T3 levels and BDNF
- Two reviewers put forward that BDNF levels were mainly driven by accessibility of human data in contrast to other consequences of TH decrease. Plausibility of an hippocampal deficit being stronger than that of BDNF reduction, shall a focus on hippocampus and/or cortex be preferable?
- T4 decrease in neuronal tissue and BDNF reduction is "weak". Should it be integrated within the KE "Altered gene expression"?

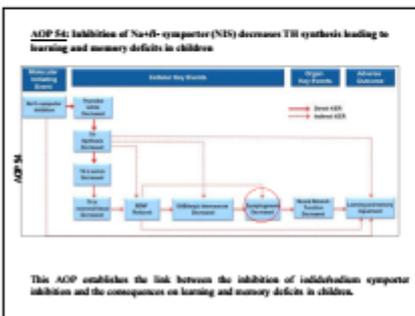
Missing literature in discussion limitations of some effects.

KE 381 and KER 444

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- Two reviewers put forward that BDNF levels were mainly driven by accessibility of human data in contrast to other consequences of TH decrease. Plausibility of an hippocampal deficit being stronger than that of BDNF reduction, shall a focus on hippocampus and/or cortex be preferable?
- Given the revisions provided should it be branched?
- T4 decrease in neuronal tissue and BDNF reduction is "weak". Should it be integrated within the KE "Altered gene expression" or should it go from weak to moderate or stand as it is?

Missing literature in discussion limitations of some effects.



TH levels can have influence beyond synaptogenesis (neuronal migration, dendritic arborization, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, hippocampus neurogenesis/volume, and callosal zone projections).

Suggestion made on adding an indirect KER as events other than synaptogenesis can modify BDNF levels.

Reply: In the paper by Robichaux et al., 2014 different pathways that regulate synaptogenesis are described; however, in this paper it is not discussed which pathways involved in synaptogenesis are under the control of TH signalling

Other issues

- Change life stage applicability from moderate to strong_ done
- Regarding the presentation on harmonization and redundancies suppressions are needed
 - Authors said they will remove when unnecessary (Note that KE [and KER] should be seen as independent documents)
- To be discussed p8, p11, p27,p33, p34,
Ex: Reorganizing KE and KERs for which *in vitro*, *in vivo* as well as epidemiological data are mixed together.

Regulatory aspect



AOPs are modular

- KEs and KERs are shared by multiple AOPs
- No need to use the same descriptions over and over
- Reusability (best practices)

AOPs are living documents

- KE and KER descriptions can be expected to evolve over time
- No descriptions are updated and expanded - all AOP descriptions they link to update automatically

AOP networks for prediction

- Entry of site based information in KE allows for the facile assembly of AOP networks.

Opinion on regulatory applicability:

Before the revision sent may 16th divergent opinions arose as to the applicability of the AOP for regulatory purposes.

One reviewer considered that this AOP was not ready to be used for regulatory purposes. One of the major gaps being the need for BDNF quantifications.

Two reviewers estimated that due to the well established KE a dynamic update of the AOP would allow for regulatory applications in the near future. The last reviewer shared this point of view and reminds that effects of "iodine supplementation" is not known and strengthens other reviewers' comments about the importance of thyroid status at the moment of NIS inhibition (MIE).

Conclusions

Given recent literature addition supporting BDNF quantification and massive revision addressing most if the points raised.

Do we agree on the fact that conclusions need to be changed .

Do you see anything we missed?

Next:

- 1) compilation of today's discussion and decisions in the final report
- 2) As revision is done by the authors, final check by everyone of the report before sending it to OECD and
- 3) will be discussed at the annual meeting in June

- Submission for endorsement to the Working Group of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA)
- Declassification by Joint Meeting and publication in the [OECD Series on AOPs](#)



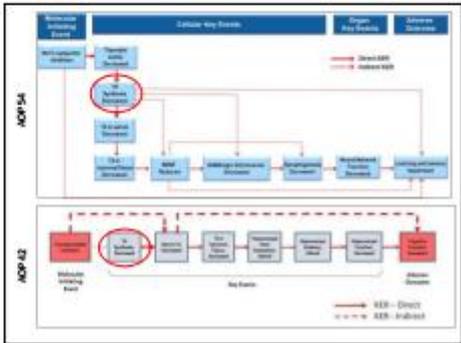
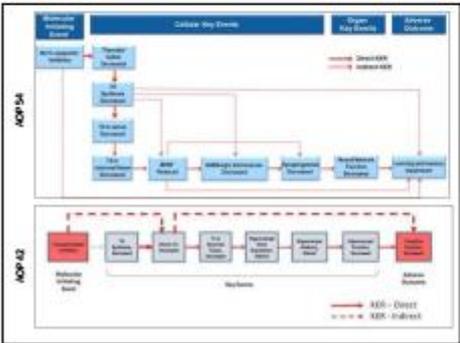
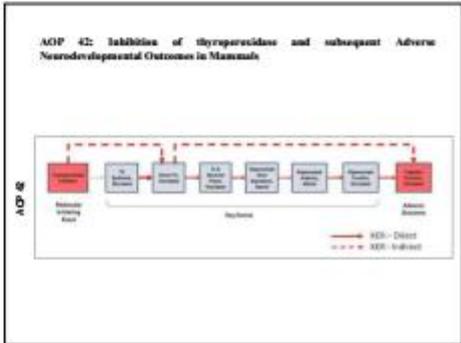
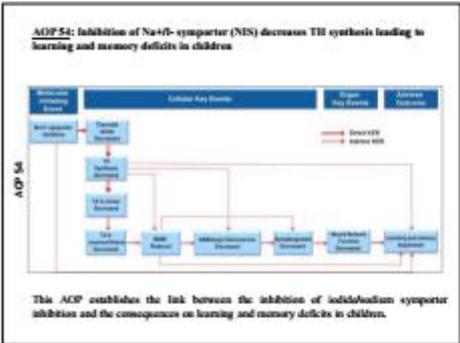
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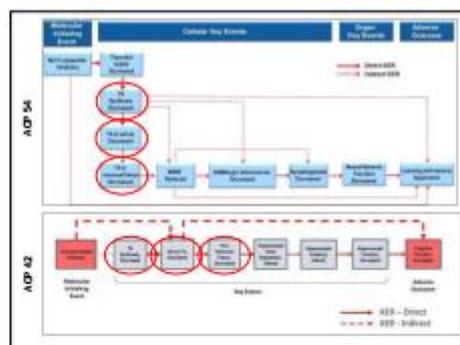
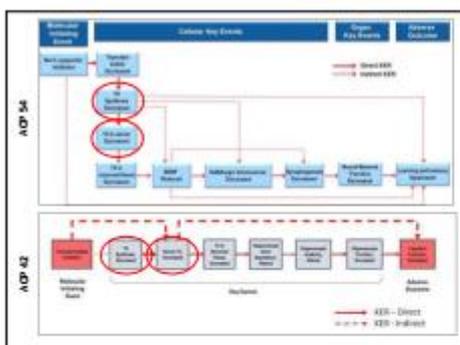
AOP54/42 overlapping issues

End of review TC
AOP 42 and 54

Overlapping issues and questions

- 2:45pm -3:30pm_ Overlapping issues on the two AOPs 54 (NIS) and 42 (TPO)**
- Brief overview of both AOP 54 and 42 (Review manager)
 - Comments on common key events: TH synthesis decreased, T4 in serum decreased, TH in neuronal tissue decreased
 - How addressing AOPs sharing Key event(s) AND adverse outcome(s) with different intermediary key events?





- KE 277 TH synthesis, decreased
- KE 281 T4 in serum, decreased
- KE 280 T4 in neuronal tissue, decreased
- KE 756 hippocampal gene expression, altered
- Vs
- KE 381 reduced levels of BDNF

- T4 in serum, decreased*
- Reviewers highlighted the need to recommend some methodologies for TH measurements as there are numerous (HPLC, MS, ELISA, RIA) with different sensitivities.
 - A related point raised by two reviewers is the crucial need for standardized methodologies and ask authors to specify if one is preferred among all existing techniques and why.
 - It should be mentioned that blood sampling should be controlled for external factors such as circadian rhythms and food intake.

- T4 in neuronal tissue, decreased*
- TH levels being different within brain structures. Dissection method is crucial for reproducibility and should be clearly mentioned

Harmonization between AOPs?

