Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP 42: Inhibition of thyroperoxidase and subsequent Adverse Neurodevelopmental Outcomes in Mammals

Short title : TPO Inhibition and Altered Neurodevelopment

This AOP 42 list six key events leading from the molecular initiating event (MIE) inhibition of Throperoxidase to the decreased cognitive function (AO). The proposed AOP states that the MIE (E 279) subsequently leads to 1) a decreased TH synthesis (KE 277) which leads to 2) a decrease in T4 in serum (KE 281) which leads to 3) a decrease in T4 levels in neuronal tissue (KE 280) and 4) alters hippocampal gene expression (KE756) which in turn leads to 5) an altered hippocampal morphology (KE757) and therefore -6) a decreased in hippocampal function (KE758) to finally adversely affect cognitive function (AO).

This document is the final report established in May 2018 of an external review started in January 2018. It reflects reviewers' comments, answers from the authors and revisions planned regarding AOP42.

The review panel all agreed on the high quality of the work done on AOP42. Even before any revisions, almost all reviewers agreed that it was constructed based on the OECD Guidance and Handbook. The reviewers developed a number of suggestions and corrections that were discussed in joint meeting with the authors. This discussion between the reviewers and the authors led to good agreement on required changes. All of these changes have now been incorporated into the newly revised AOP42. We now all find that the revised AOP42 is ready for final OECD approval and for regulatory applications in a near future.

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1. Introduction and background to specific AOP

OECD AOP 42 **Inhibition of thyroperoxidase and subsequent Adverse Neurodevelopmental Outcomes in Mammals"** with the short title**:** TPO Inhibition and Altered Neurodevelopment can be found at https://aopwiki.org/aops/42.

This AOP establishes the link between the disruption of thyroperoxidase (TPO), key enzyme of thyroid hormone (TH) organization and neurodevelopmental consequences.

The authors of this AOP are :

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This AOP describes one adverse outcome that may result from the inhibition of thyroperoxidase (TPO) during mammalian development. Chemical inhibition of TPO, the molecular-initiating event (MIE 279), results in decreased thyroid hormone (TH) synthesis (KE277), and subsequent reduction in circulating concentrations of THs (KE281) and consequently a decrease in neuronal TH levels (KE 280). THs are essential for normal human brain development, both prenatally and postnatally, modulating genes critical for a normal neuroanatomical development, with subsequent effects on neurophysiology, and finally neurological function. Therefore, chemicals that interfere with TH synthesis have the potential to cause TH insufficiency that may result in adverse neurodevelopmental effects in offspring.

The hippocampus is known to be critically involved in cognitive, emotional, and memory function. The adverse consequences of TH insufficiency depend both on severity and developmental timing, indicating that exposure to TPO inhibitors may produce different effects at different developmental windows of exposure. Herein, authors discuss the implications of developmental TPO inhibition for hippocampal anatomy (KE 757), function (KE 758), and ultimately neural function controlled by the hippocampus. The biochemistry of TPO and its essentiality for TH synthesis is well known across species.

It is important to note that thyroid stimulating hormone (TSH) is not a KE in this AOP. While TSH may play a role in feedback-driven compensatory processes, it is not directly involved in brain development. The overall weight of evidence for this AOP is strong. Gaps in current understanding include the relationship of TH-dependent gene expression

and complexities of brain development. Although quantitative information at all levels of KERs is limited a number of applications of this AOP have been identified.

The graphical representation of the AOP is accessible on the figure 1

The snapshot generated at the end of 2017 was submitted for an external review.

This AOP was last updated May 2018.

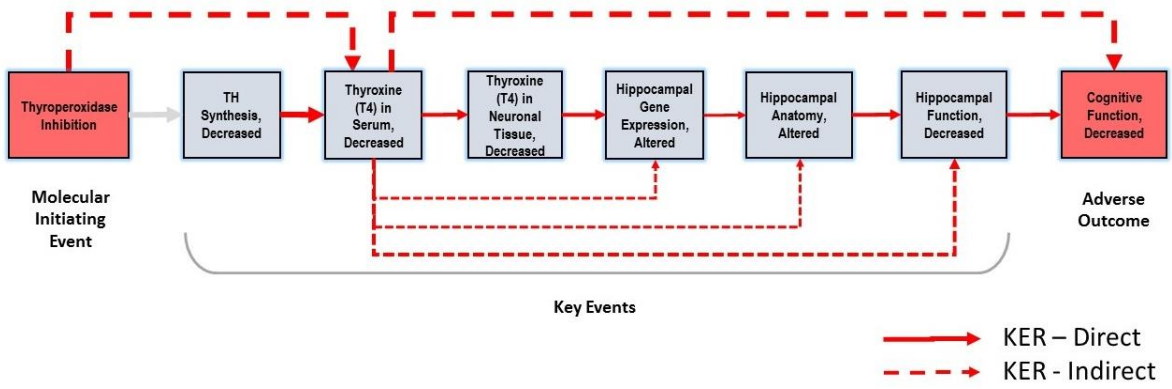

Figure 1: Graphical representation of AOP 42 Inhibition of thyroperoxidase and subsequent Adverse Neurodevelopmental Outcomes in Mammals

2. Synthesis of main issues of the review

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

AOP 42 has been reviewed during january/february 2018 by a team of 5 reviewers:

- 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)

Reviewers, were asked to reply to the following questions related to 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?*

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The version reviewed was the snapshot generated at the end of 2017.

The last update done on this AOP was done in May 2018.

2.1. Scientific quality:

Reviewers all agree on the high quality of the AOP. They acknowledge authors as experts who have published, for many of them, fundamental articles in the field of thyroid hormones and their mechanisms of regulation.
Before the TC, reviewers did not all agree on the direct application of the AOP for regulatory purposes.

Other points raised by the reviewers needed further discussion. For example, key event specificity between MIE and AO (when comparing to AOPs sharing an adverse outcome). More generally, discussion on the specificity of T4 decrease and a focus on hippocampus were needed. Further, conflicting studies were asked to be referenced or discussed.

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

Even before the teleconference and the revision of the AOP operated by the authors, all reviewers agreed on the very high quality and relevance of scientific literature cited which covered the content of the AOP and supported the biological plausibility of the pathway. All reviewers agreed that missing information comes generally from knowledge gaps and were very well presented by authors.
Classification of evidence with upstream and downstream events was justified and appropriate.
Two main points were raised. One about TPO inhibition would do not necessarily lead to a TH decrease. Second point raised was about the BDNF decrease (KE 381) stated as a key event in AOP 54. As those two AOPs share the same adverse outcome and some key events, one reviewer was wondering why not considering KE 381 in the AOP 42.

Specific points raised by the reviewers are listed below and have been discussed at the teleconference (TC). Extensive reviews were provided by the five reviewers. The complete reviews can be found in Annex 2.
Note that the order of the reviewers given in section 1 is not in accordance with the numbers given (R#) in quotations provided all along the report.

MIE _ TPO inhibition (E 279)
The multifactorial origin of TH disruption is acknowledged in this AOP (R#1, R#3). Nevertheless, the fact that other factors could balance the response of a given stressor should be discussed further.
Differences of sensitivity to decreased levels of TH depend on moment of exposure and the storage capacity of iodine and thyroid hormones. Specifically:

- Impact of Tg antibodies or age (R#1) is currently missing.
- Iodine deficiency or thyroid status at stressor exposure needs discussing :
    - R #1 highlights that many factors influence iodine intake (age, diet ..) and that differential iodine storage capacities varies with life stages should be notified in the AOP
    - R#5 reminds everyone, that strong reduction of thyroxine levels with genistein was not associated with cognitive impairment unless an iodine defect exists. R#5 also suggests to add a specific point on fetal TPO and brain/ hippocampal development (and the associated knowledge gap).

R#5 describes why the term “TPO inhibition” is misleading as it is used in a too broad sense within the AOP. Therefore, either another term is used or it should be stated that TPO inhibition also covers interactions with iodinating species.

Key Events
KE 281-T4 in serum, decrease:
R#4 highlights the need to recommend some standardized methodologies for TH measurements as there are numerous (HPLC, MS, ELISA, RIA) with different sensitivities.
R#4 asks to mention that blood sampling should be controlled for external factors such as circadian rhythms and food intake.
An important point raised by R#3 and #4 is the crucial need for standardized methodologies and ask authors to specify if one is preferred among all existing techniques and why.

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 756-Hippocampal gene expression
R#2 is questioning why BDNF expression, which is a key event in AOP54, is not referred here. Harmonization between the two AOPs is necessary. Therefore, harmonisation of KEs or why this KE is not relevant to the present AOP should be discussed at the TC.
R#3 agrees with the authors on the emphasis placed on the hippocampus given the most scientifically available literature but reminds that decreased cognitive function could have multiple origins.
R#4 states that genome wide profiles or microarray studies are not specific enough to measure the effect of TH levels on gene expression. A prerequisite on hypothyroid hippocampus gene expression would be needed.

KE 757- Hippocampus anatomy altered
R#4 mentions that the methods available to measure this KE are not specific enough. Focus must be on thyroid hormone specific alterations of the anatomy of the hippocampus. Moreover R#4 suggests to add a small section on the importance of maternal thyroid status for hippocampal development, suggestion meeting R#5 request on fetal TPO importance (see MIE).

<u>KER (Key Event Relationship)</u>
Two reviewers are calling for the inclusion of existing studies showing moderate to severe decreases in circulating TH, leading to minor or undetectable adverse effects on brain morphology in the offspring (R#2 and R#5).
Regarding decreased T4 in neuronal tissues which directly lead to altered hippocampal gene expression:
R#1 asks if any quantifiable data could be added between levels of TH in serum and in brain and gene expression.
As there is no quantitative data which supports the current KER in the AOP, “no data” instead of “weak” should be mentioned (R#5)
R#2 supports addition of an intermediate step on local deiodination as this step is crucial for altered gene expression.

TPO inhibition indirectly leads to “T4 serum, decreased”:
R#5 reminds that even with a 80% inhibition of TPO, no effects were observed on TH levels in rat.
Further, R#1 and R#3 agree with the authors on the fact that TSH levels measurements are not relevant to this AOP.

Other suggestions:
Precludes the use of “accepted dogma” or “well accepted in endocrinology” eg p39 and 62 (R#2).
Discuss non-genomic effects R#1
Use CAS numbers for the stressors to avoid any confusion due to differences in spelling (R#5)
Add a comparison of TPO across species (R#5)
TH levels are different within brain structures. Therefore the dissection method is crucial for reproducibility and should be clearly mentioned (R#4).

More details and useful suggestions are accessible within individual reviews (below and in annex2).

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 ?

All reviewers consider that weight of evidence is overall well balanced and appropriate. Uncertainties and inconsistencies are very well written and appropriately noted. The limitations of quantitative understandings of the KE are addressed.
However, two reviewers consider that WoE for the direct link between T4 decrease and hippocampal gene expression defects or anatomy is weak. R#2 suggests to add another "local conversion".
R#4 thinks that the link referred to as "moderate" by the authors for TH levels to hippocampal gene expression to hippocampus anatomy should be downgraded to "weak" as more scientific evidence is required. R#5's suggestion is to state "no data" when no quantitative data could be provided.
Conflicting views on the modifications to make regarding the evidence of key event relationships need to be discussed. R#1, R#3 and R#5 considered that the weight of evidence is appropriate, R#2 suggests to reorganize the literature cited by emphasizing TH serum levels and effects on the brain whereas R #4 highlights the need of a stronger direct causal link between T4 decrease and hippocampal gene expression or anatomical defects.

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 and the revision of the AOP, divergent opinions arose as to the applicability of the AOP for regulatory purposes. Reviewer # 1 considered that the regulatory application could be quickly achieved whereas reviewers # 2 # 3 # 4 considered that a regulatory application is limited as long as quantifications for the two endpoints mentioned by the authors are not achieved and standardized. Nevertheless, reviewers mentioned the possibility of direct applications for screening tests, either on neurotoxicity or for identification of endocrine disruptors. Reviewer # 5 was more critical of the direct applicability at the regulatory level, the idea being that a percentage of TPO inhibition is needed to trigger the cascade of events described in AOP42 and that negative cases exist and should be mentioned (in reference to the genistein case).

2.4 Conclusion:

What are your overall conclusions of the assessment of this AOP?
All the reviewers agreed on the well written and documented AOP. The gaps allowing for a strong relationship of the KERs were already identified by the authors. The suggestions mentioned by the reviewers would allow for improvement of an already very complete document. It is worth mentioning that this AOP will have to be updated regularly in order to include recent fundamental and technical knowledge that will fill the caveats due to the current state of knowledge. Discussions about the importance of quantifying /identifying threshold MIE and KE, the relationship between TPO inhibition and consequent effects on the hippocampus were needed at the TC.

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 2.45pm participants were
Reviewers: Ellen Hessel (EH), Francesca Carr (FC), Angela Leund (AL), Marta Axelstad (MA). At 2.45 Alexius Freyberger joined.
Authors AOP 54 Anna Price (AP) and Francesca Pistollato (FP) with one connection from JRC.
Authors AOP 42 Kevin Crofton (KC) and Mary Gilbert (MG)
Chair: Jean-Baptiste Fini (JBF)

Note that **reply from the authors** during the TC are in **bold**.

The dedicated call started at 2.45pm with overlapping issues between AOP42 and 54. Nevertheless AOP42 authors participated from the beginning when discussing the AOP54 specific points (2pm)

Importantly, when discussing the BDNF related KE in specific AOP54points, **KC and MG agreed with AP who discussed the fact that AOPs are all different and they could have common KE with different connections at one moment. They also mention the AOP network organization which will take place within the ongoing process:**

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 a 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 network 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.**

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.

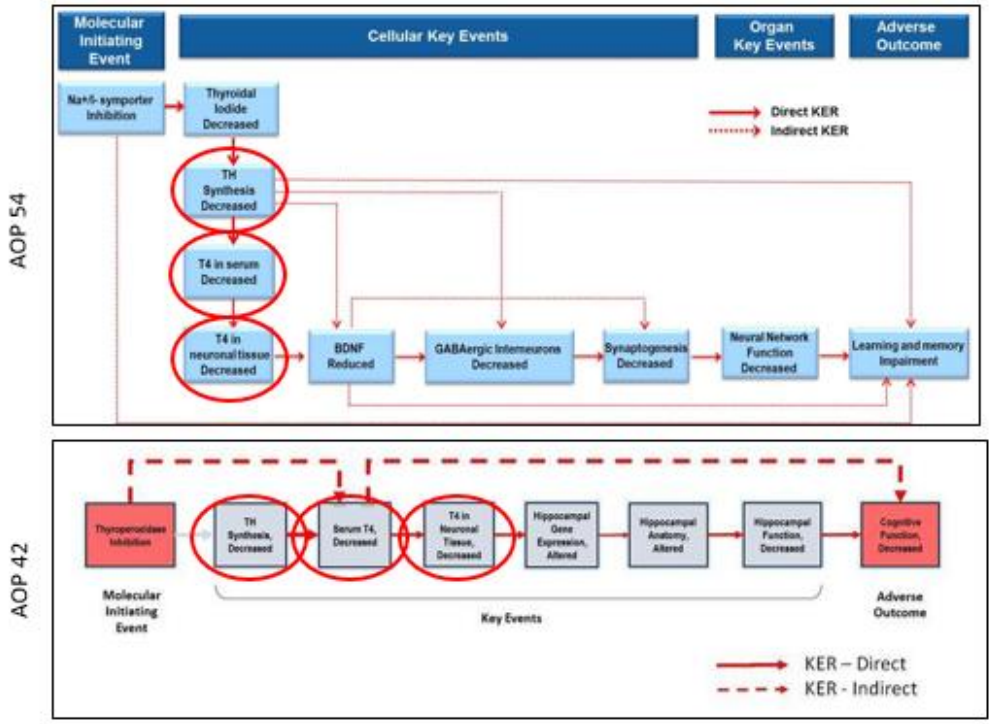


Figure 2: Comparison of the two graphical abstracts if AOP42 and54. Red circles show common KEs between the two AOPs. Note that the AOP42 graphical abstract is truncated and is not the final version (missing indirect KERs)

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 previous discussion this point was 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, **we all decided that this was not the role of authors to promote a technique even though everybody agreed on the relevance of the issue raised.**

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 that 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**.

Second part of the TC

Then, at 3.30pm specific issues were raised on AOP42.

KC and MG deeply thanked the reviewers for their hard work on the reviews which make the AOP improve.

JBF used slides to support discussion on the points raised by the 5 reviewers (see Annex 4 for the slides)

All reviewers agreed on the high quality of the AOP42 provided. KC and MG deeply thanked the reviewers for their work and suggestions on the AOP which will definitively improve the document.

Main issues discussed:

1) the "genistein effect" on TPO with no subsequent effect on cognitive impairment to be discussed within the AOP
2) Addition of a new KE: local deiodination decreased

MIE Thyroperoxidase inhibition.

During the review process R#5 raised the point that Denomination TPO inhibition is considered to be used in a too broad sense. Authors agreed with the suggestion made to modify the AOP in incorporating that TPO inhibition also covers TPO-iodinating species

Still on the MIE, others suggestions were made to consider confounding effects on TPO effects. Namely: Given that sensitivity to decreased levels of TH depends on moment of exposure and the storage capacity of iodine and thyroid hormones. Reviewers suggested to add in the KE description :

- Impact of Tg antibodies or age

And to discuss in more details:

- factors influence iodine intake (age, diet ..) differential iodine storage capacities varies with life stages
- reduction of thyroxine levels with genistein were not associated with cognitive impairment unless an iodine defect exists.

Authors did not fully agree with these suggestions. While they agree that there is evidence that iodine and antibody status can impact thyroid hormone synthesis and homeostasis, they disagree that these items should be addressed in this AOP as AOP is not designed for iodine deficiency or any other of the multitude of dietary conditions (e.g., iron, selenium) or other factors external to the KEs in this AOP.
For the genistein they agreed that this specific example should be mentioned.

- Direct fetal TPO inhibition and consequent brain/ hippocampal development in human should be mentioned (and the associated knowledge gap).

Authors agreed to integrate the changes in the AOP. They will do it at MIE, but also in uncertainties and inconsistencies for two KERs :TPO and serum TH but also serum T4 and hippocampal anatomy.

KE *Hippocampal gene expression*

- R#3 agrees with the authors on the emphasis placed on the hippocampus given the most scientifically available literature but reminds that decreased cognitive function could have multiple origins.
- R#4 states that genome wide profiles or microarray studies are not specific enough to measure the effect of TH levels on gene expression. A prerequisite on hypothyroid hippocampus gene expression would be needed.

Authors agreed to take in consideration these comments and add a comment in the AOP.

KER TPO and T4 serum decreased

Genistein case: In Chang & Doerge 2000 and Doerge &Chang 2002, genistein administration was shown to lead to 80% inhibition of TPO activity but no effects observed on TH levels in rat.

This was asked to be added in uncertainties and inconsistencies. The authors agreed with this comment from the reviewer and altered the text accordingly, as suggested.

KER T4 serum decreased and cognitive function decreased

Two reviewers are calling for the inclusion of existing studies showing moderate to severe decreases in circulating TH, leading to minor or undetectable adverse effects on brain morphology in the offspring.

Authors agreed that quantitative relationship between decrease of thyroxine and a neurodevelopmental adverse outcome is poorly understood. There are insufficient data to describe quantitative relationships owing not only to the state of knowledge of TH role in hippocampal morphology and the requisite contributions from maternal, fetal, neonatal sources when specific phases of hippocampal neuroanatomical development occur.

Therefore, authors suggested to add a comment in that sense in the AOP.

New Key event to be added Between T4 in neuronal tissue and hippocampal gene expression:

- Suggestions to add the KE related to local deiodination decreased (KE 1002?). Authors agreed that deiodinases play an essential role in maintaining appropriate concentrations of TH in brain and other organs. But they also remind that there is no "complete AOP": For this AOP there could be multiple steps added for complete TH synthesis pathway, as well as steps for release from thyrocytes, uptake from blood into tissues via cellular transporters, interaction with serum binding proteins, etc. _**Authors planned to introduce a description of the importance of deiodinase in the AOP but not include a new KE.**
- Quantifiable data should be added between levels of TH in serum and in brain and gene expression. _**Authors agreed on amending the AOP42 on that specific point.**
- If no quantitative data supports the current KER in the AOP, there is a suggestion to mention "no data"_ **This is not an option in the AOP Wiki as strength indicators are dictated by OECD's AOP Handbook and guidance document.**

Regulatory considerations

Before discussing the last part on regulatory aspect, JBF reminded everyone that AOP are living document. As methods for observing biology evolve, new possibilities for KEs arise. Importantly, there is no objective of a "complete AOP"

Before the TC, divergent opinions arose as to the applicability of the AOP for regulatory purposes.

As a reminder : Reviewer # 1 considered that the regulatory application could be quickly achieved, reviewers # 2 # 3 # 4 considered that a regulatory application is limited as long as quantifications are not achieved and standardized. Reviewer # 5 was more critical of the direct applicability at the regulatory level, the idea being that a percentage of TPO inhibition is needed to trigger the cascade of events described in AOP42.

At the end of the TC, thanks to authors reply and planned revisions all agreed on the direct applicability of the AOP 42 for regulatory purpose.

At the end of the TC, and a consensus agreement was found in planning to do the revisions discussed in short time frame. Action list was given.

No further questions. Authors thanked again reviewers and review manager for the work on review and TC.

3.3 Action list

There were actions to be done in the following days to be able to send the final report at the OECD.

1) JBF: to send the slides to everyone
2) KC and MG and answer the mains issues raised by the reviewers and to provide a revised AOP (see section 4). Specifically:
    a. Add a discussion on genistein case
    b. Add a discussion on deiodinase function in neuronal tissue
3) All: to agree on the revisions
4) KC and MG to incorporate the modifications on AOP wiki

4. Summary of planned revisions

Following the TC authors provided a detailed table answering point by point to the main issues raised and discussed at the TC.

Response to Reviewers Comments		
Section 1: Comments from Slides and Discussion during Conference Call		
Reviewer Comment	**Author Response**	**Changes to AOP42**
Denomination TPO inhibition is considered to be used in a too broad sense along the review. There is a suggestion to mention that TPO inhibition also covers TPO-iodinating species.	Authors agree and thank Reviewer for suggestion. Term was invoked as It has normally been applied in the toxicology literature, especially recent high-throughput screening.	Page 17 revised doc following has been added: *It is important to note that TPO is a complex enzyme and that has two catalytic cycles and is capable of iodinating multiple species (Divi et al., 1997). Alterations in all of these events are not necessarily covered by some of the commonly used assays that measure "TPO inhibition" (e.g., guaiacol and AmplexUltraRed, see below). Therefore, in the context of this AOP we are using TPO inhibition not in the classical sense, but instead to refer to the empirical data derived from the assays commonly used assays to investigate environmental chemicals.*
Given that sensitivity to decreased levels of TH depends on moment of exposure and the storage capacity of iodine and thyroid hormones. Reviewers suggest to add/discuss in more detail in KE description • Impact of Tg antibodies or age • factors influence iodine intake (age, diet ..) differential iodine storage capacities varies with life stages	While we agree that there is plentiful evidence that iodine and antibody status can impact thyroid hormone synthesis and homeostasis, we disagree that these items should be addressed in this AOP. The AOP is not designed for iodine deficiency or any other of the multitude of dietary conditions (e.g., iron, selenium) or other factors external to the KEs in this AOP. For genistein – please see comments below in response to slide 5.	No changes to be made.

• reduction of thyroxine levels with genistein were not associated with cognitive impairment unless an iodine defect exists.		
Direct fetal TPO inhibition and consequent brain/ hippocampal development in human should be mentioned (and the associated knowledge gap).	Agree this is an important point to clarify. The direct action of TPO inhibitors on the fetal as well as the maternal thyroid gland has been addressed in three places in the revised document	Page 18 – MIE – Uncertainties and Inconsistencies. *It is noted that prior to the onset of fetal thyroid function, TH are still required, the fetus relying solely on maternal sources. Chemical-induced TPO inhibition can affect synthesis in the maternal gland and in the fetal gland.* Page 74 – Uncertainties and Inconsistencies KER TPO inhibition and serum TH. *The relationship between maternal and fetal levels of hormone following chemically-induced TPO inhibition has not been well characterized and may differ based on kinetics. Reductions in serum TH in the fetus, in rat and human is derived a chemical's effect on the maternal thyroid gland as well as the fetal thyroid gland.* Page 118 – Uncertainties and Inconsistencies in indirect KER Serum T4 and Hippocampal Anatomy. *The role of direct fetal TPO inhibition contribution to fetal TH and subsequent changes to hippocampal structure and subsequent downstream KEs in humans is a knowledge gap.*
Hippocampal gene expression R#3 agrees with the authors on the emphasis placed on the hippocampus given the most scientifically available literature but reminds that decreased cognitive function could have multiple origins.	Agreed that hippocampus is not the only structure underlying cognitive function, the authors did not mean to suggest so. We point to several places in the document where regional specificity of brain function is mentioned that underscore that cognition is multifaceted Pg 47: Through the interconnectivity within the hippocampus and its connections to amygdala, septum and cortex, the hippocampus plays a pivotal role in several learning and memory processes, including spatial behaviors	We have added the following disclaimer to the summary section of the document to emphasize this point. Page 127. Indirect KER SerumT4 to Cognitive Function *Clearly the brain circuitry controlling cognitive function is complex and is not solely accomplished by the functionality of the hippocampus. However, it is well documented that normal hippocampal structure and physiology are critical for the development of cognitive function.*

	Pg 56: Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition of, or retrieval of, a learned event, the hippocampal-based memory systems have received the most study. Pg 105. However, the causative relationship of which specific alterations in synaptic function are associated with specific cognitive deficits is difficult to ascertain given the many forms of learning and memory, and the complexity of synaptic interactions in even the simplest brain circuit.	
Hippocampal Gene Expression R#4 states that genome wide profiles or microarray studies are not specific enough to measure the effect of TH levels on gene expression. A prerequisite on hypothyroid hippocampus gene expression would be needed.	While we agree that measurement of gene levels is not indicative of changes in protein level, the data in this arena is scarce and we include all that is available. The data included in the Table on page 109 of Word document describes gene transcripts identified in fetal and neonatal brain in hippocampus, cortex and cerebellum. Although derived from wide genome profile searches, most were from rtPCR of individual transcripts. Of those identified by microarrays, the individual transcripts of interest were typically among those followed up by PCR for validation purposes.	On TC in discussion with R# 4, agreed that no changes were needed to address genomewide profiling.
In Chang & Doerge 2000 and Doerge &Chang 2002, genistein administration was shown to lead to into a 80% inhibition of TPO activity but no effects were observed on TH levels in rat. **This should be added in uncertainties and inconsistancies**	The authors agree with this comment from the reviewer and altered the text accordingly, as suggested	Added to page 63. Uncertainties and Inconsistencies. *It is important to note that data from studies on genistein highlight this uncertainty. Doerge and colleagues have demonstrated that for this comund up to 80% TPO inhibition did not result in decreased serum T4 in rats (Doerge and Chang, 2002). This is not consistent with*

		other prototypical TPO inhibitors (e.g., PTU, MMI). It remains to be determined if for some presently unknown reason, that genistein is an outlier.
Two reviewers are calling for the inclusion of existing studies showing moderate to severe decreases in circulating TH, leading to minor or undetectable adverse effects on brain morphology in the offspring Quantitative relationship between the necessary decrease of thyroxine levels in dams, the resulting decrease of throxine in fetal brain and a neurodevelopmental adverse outcome is poorly understood and compounds with similar effects on thyroxine levels do not necessarily result in a comparable or in an adverse outcome at all (e.g., PTU versus ETU, Mancozeb Case Study, European Commission, 2017). The different outcomes for PTU vs ETU are currently not understood, whether iodothyronine deiodinase type I inhibition by PTU might play a role remains open (European Commission, 2017). Such a case should be included in the AOP and discussed.	We agree with the reviewers that the quantitative relationship between serum thyroxine and adverse neurodevelopmental outcomes is poorly understood. There are insufficient data to describe quantitative relationships owing not only to the state of knowledge of TH role in hippocampal morphology and the requisite contributions from maternal, fetal, neonatal sources when specific phases of hippocampal neuroanatomical development occur. One study is cited on page 117 of existing document in which standard morphometric indices of hippocampal size and volume were not detected at lower doses despite subsequent evidence of gene expression changes and neurodevelopmental defects in littermates. *From Page 117 Indirect KER Serum T4 to Hippocampal Anatomy - Dose-Response Evidence:* There are limited data available to inform the dose-dependent nature of the correlation between serum THs and changes in hippocampal anatomy. Gilbert et al (2007) demonstrated dose-dependent declines in the expression of protein marker inhibitory neurons in both hippocampus and neocortex with graded exposures to PTU and resultant serum T4. Shiraki et al. (2014; 2016) report dose-dependent alterations in the expression patterns of several neuronal and glial protein markers in the hippocampus after developmental exposure to different doses of PTU or MMI. Gilbert et al. (2016) report dose-dependent reductions in linear morphometry and volume of hippocampal subfields following	Added to Uncertainties and Inconsistencies in Indirect KER page 117 we have added: *In one of the few dose-response studies assessing hippocampal anatomy, alterations in simple guideline metrics of linear morphometry and volume of hippocampal subfields following developmental exposure to the PTU were largely restricted to the high dose group, despite alterations in downstream KEs of hippocampal physiology and cognitive function. This may result from inadequacy of the assessment tools or the timing of the observations. Similarly, in chemically induced serum hormone reductions of comparable magnitude as those induced by PTU or MMI, observations of hippocampal morphology are not always seen (PTU vs ETU or mancozeb, European Commission, 2017). Consideration of the sensitivity of neuroanatomical and neurobehavioral method used, as well as chemical kinetics that drive the reduction of maternal, fetal, or neonatal TH reduction, may be key to understanding these discrepancies.* Added to Appendix Table: *Some inconsistencies may arise when using maternal serum to predict offspring outcome on hippocampal anatomy if the kinetics of the chemical to not sufficiently reduce maternal hormones at the appropriate time, not cross the placental barrier to sufficiently disrupt fetal hormone synthesis, or are not sufficiently available to the nursing pup via the milk (European Commission, 2017)*

	developmental exposure to the PTU.	
"reduction of thyroxine levels with genistein were not associated with cognitive impairment unless an iodine deficiency exists'	Discussion with the Lead Reviewer stated that this comment was a cut and paste error and there is not paper describing this scenario	No changes needed
Suggestions to add the KE related to local deiodination decreased quantifiable data should be added between levels of TH in serum and in brain and gene expression.	The Reviewer is correct that deiodinases play an essential role in maintaining appropriate concentrations of TH in brain and other organs. We agree that this AOP, as well as most AOPs, have 'missing' steps. For this AOP there could be multiple steps added for complete TH synthesis pathway, as well as steps for release from thyrocytes, uptake from blood into tissues via cellular transporters, interaction with serum binding proteins, etc. We have included acknowledgement of the importance of deiodinase in the TH regulatory system several places in the current document, specifically addressing deiodinases in KER _T4 in Serum to T4 in Neuronal Ttissue. Page 37: Within the astrocyte, T4 is converted into T3 via the local activity of deiodinase 2 (DIO2) (Guadano-Ferraz et al., 1997). A small amount of T3 may cross the blood brain barrier directly via the T3-specific transporter, MCT8 (Heuer et al., 2005). Although in mature brain T3 derives partially from the circulation and from the deiodination of T4, in the fetal brain T3 is exclusively a product of T4 deiodination (Calvo et al., 1990; Grijota-Martinez et al., 2011). In both cases, only the required amount of T3 is utilized in neurons and the excess is degraded by the neuron-specific deiodinase DIO3 (Tu et al., 1999; St. Germain et al., 2009; Hernandez et al., 2010). Both deiodinase and transporter expression in brain peak in different	To address the Reviewers concern, the following sentences were added to the text on page 82 under Uncertainties and Inconsistencies: *The role of local deiodination is an uncertainty.* *In addition, future work on cellular hormone transport mechanisms and deiodinase activity is likely to inform the addition new KEs and KERs between serum and brain T4.* And to Appendix I Summary Table 2. *Other uncertainties include: compensatory mechanisms can influence local neuronal availability of T4 and these relationships have not been clarified; and there are other know steps between serum T4 and brain T, including cellular transporters and local tissue deiodinases.*

	brain regions at different times in fetal and neonatal life (Kester et al., 2004; Bates et al., 1999; Muller and Heuer, 2014; Heuer, 2007). Collectively, these spatial and temporal patterns of transporter expression and deiodinase activity provide exquisite control of brain T3 available for nuclear receptor activation and regulated gene expression. Page 80: In astrocytes, T4 is then deiodinated by Type II deiodinase to triiodothyronine (T3) (St Germain and Galton, 1997), which is then transported via other iodothyronine transporters (e.g., monocarboxylate transporter) into neurons (Visser et al., 2011). While some circulating T3 may be taken up into brain tissue directly from blood (Dratman et al., 1991), the majority of neuronal T3 comes from deiodination of T4 in astrocytes. Decreases in circulating T4 will result in decreased brain T3 tissue concentrations. It is also known that Type II deiodinase can be up-regulated in response to decreased T4 concentrations to maintain tissue concentrations of T3 (Pedraza et al., 2007; Lavado-Autric et al., 2013; Morse et al., 1986), except in tanycytes of the paraventricular nucleus (Fekete and Lechan, 2014).	
If no quantitative data supports the current KER in the AOP, there is a suggestion to mention "no data"	This is not an option in the AOPWiki as strength indicators are dictated by OECD's AOP Handbook and guidance document.	Quantitative calls remained as 'Weak' where no quantitative data are available
Quantifiable data should be added between levels of TH in serum and in brain and gene expression.	There is a data gap for quantitative understanding of hormones in serum and hormones in brain and for hormones in brain and gene expression in hippocampus. We have revised the AOP on page 83 on to address this.	*Page 83 KER serum T4 to neuronal T4 – Quantitative Understanding of Linkage. Standardization of analysis for these KEs is crucial to allow comparisons to be made between independent experiments and better judge the effects of changes in TH levels in serum and brain. Due to very low levels in brain, regional specificity of TH are not feasible in rodent studies with current detection methods.*

Hippocampus anatomy altered R#4 mentions that the methods available to measure this KE are not specific enough. Focus must be on thyroid hormone specific alterations of the anatomy of the hippocampus.	The authors disagree for a number of reasons. An AOP is not supposed to be a "how-to" paper, but instead a review of what is known. Proscriptive information of dissection techniques or specific anatomical preparations are not within the scope of the AOP. Additionally, the Handbook explicitly states that each KE is to be independent described and standalone, so descriptions of "TH-dependent" hippocampal neuroanatomical alterations in the KE section are not warranted. Finally, as with many of the KEs within this AOP, the type of anatomical change observed will depend on the severity, duration, timing of the TH reduction. Some phenotype may derive from maternal TH insufficiency, others from maternal and fetal, others from neonatal, and some may require sufficient TH deprivation over the fetal and neonatal time periods.	There appeared to be a general consensus on the teleconference of the authors' response and that no changes are needed.
Hippocampus anatomy altered There is a suggestion to add a small section on the importance of maternal thyroid status for hippocampal development, suggestion meeting request in MIE section on fetal TPO importance.	Authors agree that maternal and fetal as well as neonatal hormonal status may impact hippocampal anatomy. In responding to point from Slide 3 above this comment was addressed	The following text was added to the revised document on page 6 in the Overall Assessment of the AOP under the Domain of Applicability – *Page 6: Life stages:. The influence of maternal thyroid status prior to onset of fetal thyroid function is an important consideration.* *Page 18. Under MIE and ontogeny of TPO function.* *It is noted that prior to the onset of fetal thyroid function, TH are still required, the fetus relying solely on maternal sources. Chemical-induced TPO inhibition can affect synthesis in the maternal gland and in the fetal gland.* Page 118 – Uncertainties and Inconsistencies in indirect KER Serum T4 and Hippocampal Anatomy. *The role of direct fetal TPO inhibition contribution to fetal TH and subsequent changes to hippocampal structure and subsequent downstream KEs in humans is a knowledge gap.*

		Page 74 – Uncertainties and Inconsistencies KER TPO inhibition and serum TH. *The relationship between maternal and fetal levels of hormone following chemically-induced TPO inhibition has not been well characterized and may differ based on kinetics. Reductions in serum TH in the fetus, in rat and human is derived a chemical's effect on the maternal thyroid gland as well as the fetal thyroid gland.*
As it currently stands, divergent opinions arise as to the applicability of the AOP for regulatory purposes.	We are in general agreement with comments of the reviewers.	As this section is 'Optional', authors have deleted it from Page 12 of the document
BDNF is a KE in AOP54, but not mentioned at all in AOP 42. Why is BDNF not mentioned here?	Our approach was to examine TH-responsive genes in the hippocampus to identify the KE of this AOP. Although many transcripts have been reported, and we include a table of them in our document, it was our scientific judgement that none of them was sufficiently robust to ascertain it as pivotal for our progression in the AOP. Effects of TH disruption on BDNF have been equivocal and inconsistent. *(see review by Gilbert and Lasley (2013) Developmental thyroid hormone insufficiency and brain development: a role for brain-derived neurotrophic factor (BDNF)? Neuroscience 239:253-70.)*. BDNF does many things in the brain at different times in different regions. In our opinion, it's link to TH was not sufficiently well documented to serve as a KE.	This was discussed on the teleconference and agreed that AOP42 can stand without invoking the critical effects of TH decrements on BDNF

These changes have been submitted by the authors May 25th 2018 to the reviewer panel and the reviewer manager. Authors also sent a new version of AOP with changes incorporated. All agreed that these changes were sufficient and that the AOP should be changed following these planed modifications.

Authors did make the modifications and incorporated them in the AOP wiki first week of June 2018.

5. Further discussion

From the review manager point of view, expectations in terms of reviewing, communication, organisation and reporting are now 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 all agreed on the high quality of the work done. Before the revision almost all reviewers agreed on the next applicability of the AOP for regulatory purposes. The reviewers developed a number of suggestions and corrections that were discussed in joint meeting with the authors. This discussion between the reviewers and the authors led to good agreement on required changes. All of these changes have now been incorporated into the newly revised AOP42. All find that the revised AOP42 is 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 42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	https://aopwiki.org/aops/42 Snapshot: [HTML]; [PDF] Internal review AOP 42	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

AOP 42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals

GENERAL:

This AOP is very clearly delineated with appropriate supporting scientific evidence. The direct and indirect relationships of the key events are well documented with excellent descriptions of the physiological relevance. The essentiality of the key events and the weight of evidence are convincing and logically described. There are no substantive changes recommended to this AOP.

SCIENTIFIC QUALITY:

Overall AOP incorporates relevant scientific literature to support the relationship between the MIE, inhibition of TPO, reduction of neuroanatomical development and neurological function. The evidence provided supports the biological plausibility of the pathway and associated classification of the stressors. Numerous studies across species, including humans, have demonstrated that inhibition of TPO directly reduces TH synthesis and serum thyroxine and corresponding decreases in various regions of the brain. The evidence is strong that reduced serum T4 alters hippocampal gene expression.

MOLECULAR INITIATING EVENT

Disruption of TH synthesis may be a result of several different MIEs including dietary factors, iodine, inhibition of iodide transport through NIS, and direct inhibition of TPO. That differential storage of TH precursor and iodine varies with life stages as well as species is recognized in this AOP. The impact of a reversible TPO inhibitor will differ between adults with high storage capacity and neonates with low storage capacity. The impact of TG antibodies or age at MIE exposure could be more specifically addressed here as well as at Key Events. Should the importance of THRs in mediating TH action in the brain be noted throughout this AOP? There is not a discussion of non-genomic actions of TH in cell signaling in the brain. Aren't these key events in mediating hippocampal development and gene expression?
e.g.:

- Brent GA. 2012 Mechanisms of thyroid hormone action. J Clin Invest 122:3035-3043
- David PJ, Goglia F, Leonard JL. 2016 Nongenomic actions of thyroid hormone. Nature Reviews Endocrinol. 12:111-121.
- Flamant F, Gauthier K, Richard S. 2017. Genetic investigation of thyroid hormone receptor function in the developing and adult brain. Curr Topics Develop Biol. 125:303-335.

The classification of the evidence is appropriate.

KEY EVENTS

- The classification of the evidence in support of upstream event(s) leading (directly/indirectly) to downstream event(s) is justified adequately. AOPs referencing the relationship of TPO inhibition leading to decreased TH synthesis and subsequent adverse neurodevelopmental outcomes in mammals is consonant with the key events identified subsequent to inhibition of NIS. The key event relationships are strong.

- 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

KEY EVENT RELATIONSHIPS

1. The KERs of TH synthesis, serum T4 and brain concentrations of T3/T4 are particularly well described with appropriate supporting evidence.

2. Since this AOP was developed, additional studies further strengthen the relationship between thyroid hormone levels and neurogenesis and hippocampal development.

- The impact on hippocampal neurogenesis of impaired thyroid during developmental but not adult periods emphasizes age-related vulnerabilities.

Gilbert ME, Goodman JH, Gomez J, Johnstone AFM, Ramos RL. 2017 Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption. NeuroToxicology 59: 9-21.

- 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. A recent review of TH signaling and neurogenesis across species may strengthen this KER.

Gothie J-D, Demeneix B, Remaud S. 2017 Comparative approaches to understanding thyroid hormone regulation of neurogenesis. MCE 459:104-115.

- Bernal J. 2017 Thyroid hormone regulated genes in cerebral cortex development (Review). J Endocrinol 232:R83-R97.

Overall, the uncertainties or inconsistencies are appropriately noted. The limitations of the quantitative understanding of the KE linkages are addressed.

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 TPO activity, TH levels and neurodevelopmental outcomes, it is highly probable that it will have broad regulatory applicability. This AOP can provided 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. Inhibition of TPO can be reversible. Thus, the timing of exposure, the length of exposure should be considered in any regulatory framework.

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 #2

OECD, AOP External review: Reply to charge questions

In general, a lot of hard and very relevant work has been put into the making of these two AOPs. I however find that it weakens the concept and regulatory use of the AOP concept when two AOPs which both have a common KE (low TH in serum) and the same AO (impairment in learning and memory) are so different and do not share the same (or only very few of the same) KEs and KERs. I find that this issue should be discussed further in the review group and with OECD.

Below, please find my review of AOP 42.

AOP 42: Inhibition of thyroperoxidase and subsequent Adverse Neurodevelopmental Outcomes in Mammals

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?

I find that this AOP incorporates almost all relevant scientific literature (of which I am aware). I can only think of a few issues which I do not find that the authors have been dealt with in enough detail.

1) In the AOP 54, a lot of emphasis is put on the importance of BDNF (levels and expression) in relation to neurological development. Many of the cited papers are form the group of Dr. Gilbert, showing reduction in BDNF in developmentally hypothyroid animals. If this is indeed a common finding in hypothyroid animals and this factor is important for neurological development, I find that it would be informative to include this information in the present AOP. If, on the other hand, the authors of the present AOP (after evaluating all the available data on this endpoint), do not find this KE to be crucial for adverse brain development in relation to hypothyroidism/hypothyroxinemia, I believe that this should be better reflected in AOP 54.

2) Some places the authors state that the KERs they describe are strong, because they are 'accepted dogmas within the scientific community' or 'well accepted in endocrinology'. These statements are however not always substantiated with references or study descriptions, in the 'biological plausibility' paragraphs. That something is an "accepted dogma" is not an argument, so please revise by substantiating all these sorts of statements with study references (for instance on p 39 and 62).

3) The issue of quantitative predictive models linking serum TH concentrations to adverse cognitive outcomes is in my view not fully discussed. It is stated several times that this correlation has not been performed (or only performed for hearing loss) due to lack of studies examining this. Furthermore the authors state that "the occurrence of the final AO when upstream key events are observed is extremely consistent" (p 66). While these statements may be partially true, I believe that the authors should in this AOP also include studies from the open scientific literature, showing cases where moderate to severe decreases in circulating thyroid hormones levels in pregnant dams, only lead to minor or no detectable adverse effects on behavior or brain morphology in the offspring. Because such examples do exist but are not mentioned, it may leave the reader with the impression that all cases of developmental exposure to TPO inhibiting chemicals will cause adverse DNT effects, even though we don't know whether this is actually the case - because it has only been shown consistently with MMI and PTU.

4) The human link between thyroid hormone decrease during development and adverse neurological outcomes, and especially the aspect of quantification of T4 decreased is in my opinion not discussed in enough detail. For instance only one study (Haddow et al 1995) is cited on page 62 where this issue is discussed, even though much more literature examining the relationship between maternal T4 levels and neurological outcome in the children is present in the open literature (Haddow *et al.* 1999, Pop *et al.* 1999, Kooistra *et al.* 2006, Li *et al.* 2010, Henrichs *et al.* 2010, and more…). I find this very important, because the better the presently available knowledge from humans is described, the easier it will be to link a %-decrease in T4 (in an animal study) to a likely adverse neurological effect, without even having to perform the neurological assessment in vivo.

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 the few exceptions mentioned above.

Additionally, on page 29, in the table with scientific evidence supporting the linkages in the AOP, I find that a step is missing when the authors state that "Decreased T4 in neuronal tissue" **directly leads to** "altered hippocampal gene expression". I think that it should be reflected in the table and in the AOP description in general (possibly as a KE) that there is an important step in between (i.e. local deodination of T4 to T3 which then binds to the TRs and causes the altered gene expression). And since the action of the deiodinases can be upregulated in cases of low T4, so that more T4 is converted to T3 in order to compensate for the lower T4 availability, low neuronal T4 levels may not in all cases lead to altered gene expression. I therefore find that the "local conversion of T4 to T3 – step" should be included in the AOP description.
This issue is also discussed on p. 39, in the "weight of evidence section". Here I find that some more clear references to the studies actually measuring the link between serum TH concentration and brain TH concentrations (prior to the *empirical support for linkage* section) would add value to the description. As long as they are not provided, it is difficult for the reader to determine whether the WoE link is "moderate" and the "biological plausibility" for this KER is strong, as stated by the authors. I realize that the available empirical support is referred to in the next paragraph, but here I find that it would be useful to divide the references into those showing proportionality between the TH levels in serum and the brain, and those investigating this relationship in other tissues.

Overall, I find that the section on page 66-67 on *uncertainties, inconsistencies and data gaps* is very well written and includes many of the key uncertainties of using this AOP. I do feel that it should be specified that the adverse developmental neurotoxicity effects seen in the few cited studies using other compounds than PTU and MMI (PCB, BPA and TBBPA), may not have anything to do with changes in thyroid hormone levels during development, but could be effects occurring due to disturbances in other endocrine systems or be caused by direct neurotoxic effects of these chemicals.

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

I find that this has been very nicely explained in the present AOP in the *weight of evidence summary* on pages 65-69. I agree with the authors when they state that until we have a **quantitative** relationship showing;

1) To what extent TPO inhibition *in vitro* has to be affected in order to elicit a T4 *decrease in vivo*
2) To what extent circulating T4 levels must be decreased (in dams and in offspring at different developmental stages) before adverse effects are observable in behavior and morphological brain differentiation

we can only use QSAR or in vitro data for TPO inhibition to prioritize chemicals for further testing. This means that based on the present AOP we cannot presently predict which compounds predicted or found to act as TPO inhibitors (in QSAR models and in vitro batteries) wil indeed cause adverse effects on brain development.

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

The AOP is well written, thorough and well augmented, except for the few exceptions which I have stated previously.

Below are my smaller, additional comments for revision of this AOP:

P12. Line 12. The word "is" is missing in the sentence starting with "In serum, it is the …of the hormone that **is** thought to be …."

P 15, line 8 from bottom: please delete the word "as" in the following sentence: In the rat, either whole brain or cortex have been preferentially assessed ~~as~~ due to the low…

P19, line 6: please include a "space" in the between the words "phases" and "can", in the sentence Tables of gene clusters associated with these phasescan…

P 19,section "How is it measured". It would be helpful to include a sentence or two about how large the overlap is between gene expression profiles in the three mentioned species (humans, non-human primates and rodents). Are the same genes affected in all three species, are there no overlaps, or are we somewhere in between.

P 19,section "How is it measured". The last sentence of the paragraph is the first time in the entire section that the TH regulation of this process is mentioned. Since at least 7 of the references connected to this KE description are in studies investigating TH related effects, some more information about this in the text would improve the description.

P20-22: KE 757 is called "Hippocampal anatomy, Altered" but in reality the KE explains hippocampal anatomy, but not how it may be altered. Therefore please provide some information about the effects on hippocampal anatomy when TH levels are decreased in the KE description.

P 21, How this key event works, line 6. Pleas add an "s" in the word "it" in the sentence "Dentate gyrus forms in late gestation with most of it**s** development…

P 23-25 KE 758. There is no mentioning of the role of thyroid hormones in the section "How this key event works", and this issue is only touched upon briefly in the "How is it measured or detected" section. Please expand a little bit more regarding the role of TH in this KE.

P28 “regulatory examples using this AO”. The Bellinger 2012 paper that is referred to is in my opinion not an example of “regulatory use”. I would rather suggest to exemplify using references to regulatory reports (EFSA, ECHA, FDA EPA) where regulatory action has been taken (classification, restriction of use) based on decreased cognitive function caused by developmental exposure to a chemical. Furthermore, the next sentence is a repetition of content already stated in the previous section (p 27 line 3-6 from bottom).

P31, last paragraph before the references: It would be valuable if the authors could explain in a bit more detail what they think of this finding from the Chang and Doerge 2000 study. It this lack of in vivo effect on T4 after an 80% TPO inhibition a normal response ? Was the genistein maybe metabolized to compounds which are not TPO inhibitors or what was the explanation of the study authors to this phenomenon – and how do the AOP authors reflect upon this finding ?

P33, line 13. Please delete the “a” in the sentence: “ a Furthermore…

.

P40 “qualitative understanding of the linkage”. Is the qualitative linkage between TH levels in serum and in other tissues not shown in any of the papers cited on the previous page (empirical support section)? If this is indeed the case, please expand a little bit on this issue.

P 42, weight of evidence, last sentence. It is stated that **studies are limited**, but no references are provided. If indeed there are some studies showing this please provide citations. If no studies are available please state that this is the case.

P65, line 2 from bottom. Please add the word “is” in the sentence : It **is** also….”

A full reference list of all used references would be nice to have,

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?**

I have reviewed the AOP snapshot and responses from the authors to the previous reviewers' comments in preparation for this external review. The broad hypothesis of AOP 42 is based on the strong scientific evidence that thyroid hormone is critical for normal neurodevelopment, such that the MIE of thyroperoxidase inhibition results in several KEs corresponding to decreased thyroid hormone availability and hippocampal defects to result in the AO of decreased cognitive function (specifically the components of decreased learning and memory; and decreased cognition).

This revised AOP is well-written, comprehensive, and in a very systematic approach, addresses the complexity of the potential adverse effects of thyroperoxidase (TPO) inhibition. The authors are established scientists and regarded experts on this topic, and a significant amount of the original work on this topic has been published by several of the authors themselves.

The specified KE are comprehensive, but as the authors state, current gaps in knowledge regarding the complexities of brain development remain in this pathway. Decreased cognitive function is a relatively broad term which can refer to variety of adverse clinical measures (i.e. IQ, neuropsychological tests, behavioral tests, others). Thyroid hormone-dependent actions in the brain include neuronal migration, dendritic arborization, synaptogenesis, axonal myelination, cortical volume/cytoarchitecture, cerebellar proliferation, granule cell migration, Purkinje cell maturation, and callosal zone projections, in addition to the hippocampal neurogenesis and volume that is focused in this AOP. As such, although there are likely other KEs which can also result in the AO, the focus of this AOP on the hippocampus is one of the most scientifically developed in the available literature (both animal and human data). I also agree with the authors that thyroid stimulating hormone (TSH) action is not a KE required for this pathway, since TSH has not been understood to have any direct action on brain development, but rather on feedback within the hypothalamic-pituitary-thyroid axis.

Overall, this AOP describes what is known and what remains to be better understood regarding this pathway. The bibliography for the AOP is complete and captures the seminal references on this 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?**

Overall, I agree with the weight of evidence designations assigned to each of the KEs, KERs, and the overall AOP. The weight of evidence supporting the MIE by methimazole and propylthiouracil, in addition to other agents in the environment and diet, is high. Methimazole and propylthiouracil are medications used commonly in the clinical setting to treat hyperthyroidism, in order to reduce thyroid hormone overproduction, and have been in use as such since the 1940s. As the developers state, there is a high level of evidence that AOP 42 is most relevant during early periods of neurodevelopment, and that the effects apply to both genders.

Regarding the KERs, the literature also supports the high level of evidence stated by the developers regarding the relationships between decreased serum thyroid hormone concentrations resulting in several hippocampal gene expression and anatomy. It is well-established that decreased serum thyroid hormone concentrations also result in the AO. I agree that the current understanding regarding the other KERs is less robust and supported by a moderate weight of evidence.

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 two points 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 the necessary initial components to generate a computational model. It is reasonable to also utilize this AOP to support development of a high throughput screening assay detecting other thyroperoxidase inhibitors that have been proposed from in vitro data.

4. Conclusion

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

I believe that AOP 42 is well-prepared and has been comprehensively organized by established scientists who have the appropriate background and expertise on this subject. Although the action of TPO inhibition on the hippocampus likely represents just one of the many effects of thyroid hormone on the brain, the available strength of evidence is appropriate justification to focus on it as a mediator toward decreased cognitive function. The AO is complex, and the clinical outcomes encompassing it are broad, thus there are still uncertainties which surround this AOP. Overall, the developers have nicely captured what is currently known about this pathway.

Reviewer#4

AOP external review – 2018 – AOP 42 Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals

General comments

The AOP includes a lot of scientific and peer-reviewed literature and clearly describes the pathway from the molecular initiating effect up to organ and organism effects. It would be helpful in general to explain the functioning and mechanism of the thyroid system in the beginning of the AOP with a separate figure that include all the aspects of thyroid functioning.

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. For AOP 42 MIE "Thyroperoxidase, Inhibition"
    a. Mention T3 and / or T4 in the figure 1, that makes it more clear.
    b. For "how does this Key event works: please explain the ontogeny of TPO for rodents and humans separately. Now it is difficult to distiquish the two.
2. For AOP 42 MIE "Thyroperoxidase, Inhibition" 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*". Within this document, the TPO assay is explained at page 32-35. If this method will be (further) validated by OECD this will have preference.
3. For KE 'T4 in serum, decreased' (page 10-14) 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).
4. For KE "T4 in neuronal tissue, decreased", paragraph 'how it is measured and detected' (page 16). Based on the brain region specific levels it is important not to measure whole brain levels but also brain region specific TH levels (Constantinou et al., 2005). Please mention that the way of dissecting the brain regions is crucial to draw the right conclusions.
5. For KE 'hippocampal gene expression' paragraph 'how it is Measured and detected' (page 19): genome wide profiles or microarray studies are not specific enough to measure the effect of TH levels on gene expression. This is not a specific endpoint for this KE. First it must be studied which genes in hippocampus will be changed by hypothyroidism and then if mRNA (rtPCR) and protein levels are disrupted due to the hypothyroidism. Thereafter, the role of these altered expression levels of the specific genes on the hippocampal anatomy must be studied in more detail to be more specific within this KE.
6. For KE 'hippocampal anatomy' paragraph 'how it is measured or detected' (page 22) the methods are not specific enough. Focus must be on thyroid hormone specific alterations of the anatomy of the hippocampus (now it is only described how to measure the anatomy of the

hippocampus, but no link can be made with thyroid hormone levels and what the anatomical changes are) (Koromilas et al., 2010).

7. For AOP 42 KE 'hippocampal gene expression' and 'hippocampal anatomy' and the KER between the two:
    a. Nothing is mentioned for this KE about the role of maternal T4 levels on the gene expression and the development of the hippocampus, but the prenatal period including thyroid levels are crucial for normal hippocampal development (Moog et al., 2017; Willoughby 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?

For AOP 42 the weight of evidence

a. In the weight of evidence for the KER between 'Hippocampal Physiology Altered' leads to 'Cognitive Function' the cognitive function is mentioned as episodic memory. Please specify what the relation is between episodic memory and cognitive function.
b. a clear scientific link between thyroid hormone levels in hippocampus, hippocampal gene expression, and hippocampal anatomy is missing. Therefore, the empirical support of the two KER in between these KE are determined by the authors as moderate. The reviewer agrees with the author but even think that this is weak. Gene expression changes can in some cases affect the anatomy of the hippocampus, but it is not clear which genes are involved and how alterations in these genes affect the hippocampal anatomy. Additionally it is unclear how thyroid hormone levels in the hippocampus will affect gene expression in the hippocampus and how that will affect the hippocampal anatomy. More scientific evidence is needed. This is the weakest point of this AOP. The other KE and KER are better supported by scientific data. The scientific evidence for this AOP and the studies referring to the indirect KERs are much stronger therefore, unless these huge uncertainties between these KE, this AOP is very interesting, good designed and obvious to occur (also based on the available epidemiological evidence).

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 a decrease in cognitive functioning in mammals. 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 and important aspect of thyroid disruption for which many epidemiological evidence is available. At this stage it is still difficult to take a regulatory decision based on this AOP, since further development and more scientific data underlying the KE and KERs for the AOP are needed. As soon as it is applied for prioritization, more data will become available for the individual KEs/ KERs

4. Conclusion:

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

I would recommend this AOP for submission since 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 the mechanistic data. The AOP is very useful and obvious to occur

based on the indirect KEs. However, more research is needed on the specific effect of TH levels on gene expression in the hippocampus and how that gene expression specifically affects the hippocampal anatomy and functioning. The descriptions and alterations are not specific enough and too limited yet. Additionally, more standardization to measure TH levels is needed in future.

References:

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.

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.

Willoughby KA1, McAndrews MP, Rovet JF. Effects of maternal hypothyroidism on offspring hippocampus and memory. Thyroid. 2014 Mar;24(3):576-84. doi: 10.1089/thy.2013.0215. Epub 2013 Nov 13.

Reviewer #5

Scientific Quality

In general the pertinent literature is cited, however, in some cases conflicting papers that challenge the adverse out pathway are missing and/or need to be discussed. The current understanding of chemicals' interactions with TPO is not adequately covered, when using the term "Thyroperoxidase Inhibition" as this term – if used in a strict sense – dose not cover all of these mechanisms. Information dealing with the treatment of human fetal thyrotoxicosis and pointing to a potential role of fetal TPO inhibition should be included. Details are given below:

Event 279 - Key Event Title: Thyroperoxidase Inhibition

In the light of the current text and explanations in the AOP the title "Thyreoperoxidase Inhibition" is misleading and with regard to certain types of interaction even incorrect. An adaptation of the wording is necessary to correctly cover the current understanding of interactions with thyroid peroxidase-catalyzed reactions.

Rationale:

The biochemistry of haloperoxidases is complex. For TPO two different catalytic cycles that have one element in common are described.

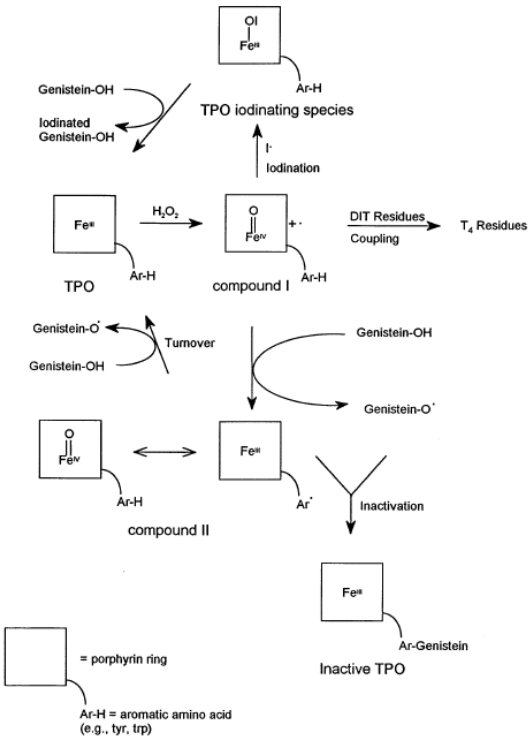
Cycle 1: TPO is oxidized by hydrogen peroxide to form Complex I. Stepwise reduction (one electron reductions) of Complex I via Complex II restores TPO in the initial state. Complex I and probably Complex II play an important role in the coupling reaction. They can also oxidize foreign compounds to radicals that can be lethal to the enzyme.

Cycle II: TPO is oxidized by hydrogen peroxide to form Complex I. Complex I then combines with iodide to form the TPO iodinating spezies (by many researchers addressed as enzyme localized hypoiodous acid). The TPO iodinating species iodinates tyrosine contained in thyroglobulin to form mono- and dioiodotyrosone.

The following ways of interaction of a chemical with TPO-catalyzed reactions are thus possible and have been described:

a) Formation of a reversible complex with TPO that inhibits TPO enzyme → reversible inhibition
b) Oxidation by complex I or II to a radical that bounds covalently to TPO and destroys TPO enzymatic activity → irreversible inhibition
c) Preferred iodination by TPO-iodinating species instead of iodination of tyrosine contained in thyroglobulin
d) Redox reaction with TPO-iodinating species, the chemical is oxidized by the TPO-iodinating species resulting in oxidized chemical, iodide and TPO in the resting state. No iodination of tyrosine contained in thyroglobulin occurs.

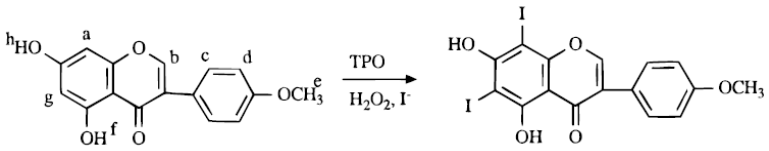
The following scheme shows the catalytic cycles and the interaction types b and c for genistein (from Diwi et al., 1997)



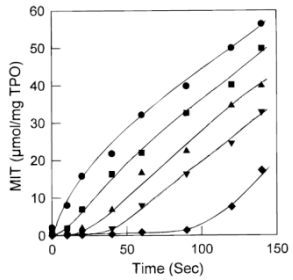
SCHEME 1. Proposed mechanisms for genistein inhibition of TPO-catalyzed reactions.

Interaction types c and d do not involve inhibition of TPO enyme, they rather inhibit the outcome of the desired reaction, namely iodination.

An example for chemicals interferring according to type c represents Biochanin A which is is an excellent substrate for iodination and is preferred by TPO-iodinating species. Iodination of biochanin A occurs exclusively as long as biochanin A is presend:



Once all Biochanin A is iodinated, iodination of tyrosine (formation of mono-iodo-tyrosine (MIT) in the experimental model shown below, re-occurs. With increasing concentrations, MIT formation is suppressed for an increasing time period. After suppression the rate of re-appearing MIT formation is comparable to the control rate (from Diwi & Doerge, 1996).



The scenario is comparable for compounds that undergo redox reactions with TPO-iodinating species such as ethylenethiourea (ETU). However, the chemical is not iodinated, but oxidized (Doerge & Takazawa, 1990)

Accordingly, interactions with TPO exist that are generally not covered by the term "enzyme inhibition" in general or "Thyroperoxidase Inhibition" in the special case. It is important in this context that in vivo (rat) propylthiourea (PTU) is considered to interact according to type d (Taurog & Dorris, 1989).

The term "Thyroperoxidase Inhibition" is thus very problematic, it may be replaced by another one, but in practical terms it may be more simple just to exlain in the beginning of the AOP that in the context of the AOP "Thyroperoxidase Inhibition" also covers interactions with the TPO-iodinating species that may not be addressed by "Thyroperoxidase Inhibition" if used in a strict classical sense.

Key event component: Iodide Peroxidase Activity – Thyroid Peroxidase – "Decreased"

Regarding above mentioned interaction types c and d, TPO enzyme activity is **not** inhibited/decreased, it is the desired outcome, thyroid hormone synthesis, which is decreased. Thus, the wording must be changed.
Proposal: Instead of "Decreased" use "**Activity Decreased or Iodinating Species Trapped**."

List of Stressors

For a given compound only one spelling should be used. Currently several options are given (e.g., ethylenethiourea and ethylene thiourea). To allow unequivocal identification CAS numbers could be given for all compounds.

Taxonomic Applicability

TPO is certainly a MIE that is conserved across taxa, however, it would also be worthwhile to address (potential) species differences regarding sensitivity to TPO inhibition. Systematic investigations in this field are urgently missing. The very limited work available, however, suggests that depending on species/class of compound huge differences may exist. Takayama et al. (1986) showed that cynomolgus monkey TPO (used as a surrogate for human TPO) was at least 450-times less sensitive towards inhibition through sulfamonomethoxine compared to rat TPO. In line with this biochemical observation sulfamonomethoxine (270 mg/kg for 5 weeks) decreased T4, increased TSH and thyroid weight and induced thyroid hyperplasia in the rat, whereas no such finding were observed in cynomolgus monkey (270 mg/kg, 5 weeks) (Takayama et al. 1986). A strong inhibition by a given compound in one species may result only in very weak inhibition in another one and would thus not result in an adverse outcome.

Event 277 - Key Event Title: Thyroid hormone synthesis, Decreased

Taxonomic Applicability

It appears to be unlogic if under event 277 less species are mentioned than under event 279.

Chemistry

Occasionally, propylthiouracil (PTU) and methimazole (MMI) are both addressed as thiouracil derivatives, however, MMI is a imidazole derivative.

Other Considerations - Kinetics

There is old data suggesting that the reason for the strong effect of thiorureylene compounds like PTU and MMI on thyroid hormone synthesis is active accumulation in the thyroid (summarized by Neary et

al., 1984). Also for other compounds active uptake into the thyroid is speculated as prerequisiste for anti-thyroid action (Weisberger, 1983). These considerations are possibly beyond the scope of this AOP, but inclusion may be helpful and could be considered.

Other Considerations - TPO Inhibition in fetal thyroid gland

Both PTU and MMI used most as pharmacological tool to decrease thyroid hormone levels have a proven history in the treatment of human fetal thyrotoxicosis, i.e., they are able to cross the placenta (Kurtoglu & Özdemir, 2017). Given the fact that these compounds are accumulated in the thyroid (Neary et al., 1984), they also should interfere with thyroid hormone synthesis in fetal thyroid. Even if the fetal thyroid in the rat is only active for about 5 days(in human it is many months) until birth it contributes to the overall thyroxine level in the fetus and a inhibition of fetal hormone synthesis should contribute to neurodevelopmental toxicity. The ability of compounds to reach the fetal thyroid or not could be an important discriminator for TPO inhibitors regarding neurodevelopmental toxicity. I am unaware of any studies dealing with this aspect, however, this knowledge gap should be addressed. Although not explicitly mentioned, the AOP rather implies effects on maternal serum thyroxine levels through TPO inhibition. Clarification is needed regarding fetal TPO inhibition.

Weight of Evidence (WoE)

In general, WoE assessments and scorings are appropriate. If quantitative data are lacking, this should be clearly stated. There are cases of inconsistency that are not discussed in the AOP. At present, there are also no rules describing how an inconsistency would affect scorings. Such an approach should be developed. Further details are provided below:

For two Key Event Relationships it is stated in the AOP that no quantitative data are available:

Thyroxine (T4) in neuronal tissue, decreased	directly leads to	hippocampal gene expression, altered
Hippocampal gene expression, altered	directly leads to	hippocampal anatomy, altered

Accordingly, regarding quantitative understanding rather “No Data” than Weak” is the proper qualifier.

There is inconsistency regarding

Thyroperoxidase, inhibition	indirectly leads to	T4 in serum, decreased

Strong inhibition of TPO activity is obviously not for all chemical classes necessarily associated with a reduction of circulating thyroid hormone (TH) levels. Following administration of genistein resulting in a reduction of ex vivo measured TPO activity by more than 80%, no effects on THs or TSH serum levels were observed in rats (Chang & Doerge, 2000; Doerge & Chang, 2002), although the rat is considered to be a very sensitive species regarding interference with thyroid function. Similarly, a diet rich in soy compared to a standard diet had little impact on biochemical and histopathological parameters of thyroid function in rats, unless combined with iodide deficiency (Ikeda et al., 2000). It remains open for the moment if genistein is a very peculiar case, or whether isoflavonoids or even resorcinol derivatives in general would be associated with a similar outcome. Anyhow, this inconsistency needs special consideration. The example of genistein may also illustrate that TPO inhibition is necessary, but not sufficient to result in decreased TH in serum and that additional conditions, e,g, iodide deficiency, are required. This aspect is also missing in the AOP.

There is also inconsistency regarding

Thyroxine (T4) in serum, decreased	indirectly leads to	cognitive function, decreased

Quantitative relationship between the necessary decrease of thyroxine levels in dams, the resulting decrease of throxine in fetal brain and a neurodevelopmental adverse outcome is poorly understood and compounds with similar effects on thyroxine levels do not necessarily result in a comparable or in an adverse outcome at all (e.g., PTU versus ETU, Mancozeb Case Study, European Commission, 2017). The different outcomes for PTU vs ETU are currently not understood, whether iodothyronine deiodinase type I inhibition by PTU might play a role remains open (European Commission, 2017). Such a case should be included in the AOP and discussed.

Regulatory applicability

In its current form the AOP is problematic when it comes to regulatory applicability. Considerable knowledge gaps exist, and the AOP is providing no guidance how to deal with that gaps.

Data indicating considerable differences in sensitivity of TPO from different species raise the question on what data regarding interactions with TPO one should rely on and make the decision "TPO inhibited" in a regulatory context. Ideally this would be data from studies using human TPO, however, such studies are rare. Studies based on rat TPO are likely to overestimate the effects compared to human TPO at least for certain compounds and whether the huge number of studies performed using hog TPO properly reflect effects on human TPO is not broadly established.

Strong inhibition of TPO, even if demonstrable ex vivo does not necessarily translate into a decrease of circulating thyroid hormone levels (the '"genistein case"). In addition, further contributing factors may be necessary (e.g., iodine deficieny in case of soy constituents). In addition it is open to what extent an additional factor would be needed (E.g., would mild iodide deficience suffice ?). Accordingly, TPO inhibition alone even if demonstrated ex vivo would be an unreliable starting point for regulatory applicability.

Quantitative relationship between the necessary decrease of thyroxine levels in dams, the resulting decrease of throxine in fetal brain and a neurodevelopmental adverse outcome is poorly understood and compounds with similar effects on thyroxine levels may not readily result in a comparable or in an adverse outcome at all (e.g., PTU versus ETU, the "Mancozeb Case" discussed by European Commission, 2017). Also the role of counter regulation in fetal brain (e.g. through iodothyronine deiodinase type II) and its capacity is not well understood.

Experimental manipulations used most to provoke neurodevelopmental toxicity are iodide deficiency and treatment with methimazole or propylthiouracil. Any of these treatments would affect maternal and fetal thyroid hormone synthesis. There is little, if any work that decribes the contribution of interactions with fetal TPO for an adverse outcome.

Accordingly, there are still considerable knowledge gaps in general and especially in quantitative terms, e.g., how much interaction with/inhibition of TPO is needed in vivo to result in a subsequent decrease of circulating hormones, are there transporters or other mechanisms available that shuttle certain compounds into the thyroid and others not, to what extent must thyroxine decrease in the maternal circulation and then fetal brain in order to result in neurodevelopmental toxicity, is there a crucial role for interactions with fetal TPO and to what extent can adaptive mechanisms compensate a toxic insult.

In my opinion, an adverse outcome only develops if the decrease of thyroxine in fetal brain exceeds a certain threshold that corresponds, possibly with some Caveat, to a threshold decrease in circulating thyroxine in dams. Neither these thresholds are clearly understood nor is there a quantitative

understanding how TPO inhibition potentially in dam and fetus would translate into a sufficient decrease of thyroxine in fetal brain.

In the light of the many knowledge gaps and quantitative uncertainty, I do not think that the AOP in its current form is ready for a broad regulatory applicability.

Conclusions

The AOP lists MIE, KEs and final adverse outcome in a logical order.

In general the pertinent literature is cited, however, in some cases papers that challenge the adverse outcome pathway are not addressed.

The KERs are not always as strong as stated in the AOP

A peculiar weakness is the unclear quantitative relationship between necessary degree of TPO inhibition, the necessary decrease of thyroxine levels in dams, the resulting decrease of throxine in fetal brain and a neurodevelopmental adverse outcome and the lack of understanding why a similar decrease of thyroxine for different compounds does not lead to the same or an adverse outcome at all.

A definition what "Thyroperoxidase Inhibition" in the context of the AOP means should be given and the definition should be such that all known mechanisms of interaction with TPO-catalyzed hormone synthesis are covered.

In the light of the many knowledge gaps and quantitative uncertainty, I do not think that broad regulatory applicability is already given for the AOP in its current form.

References

Chang HC & Doerge DR. Dietary genistein inactivates rat thyroid peroxidase in vivo without an apparent hypothyroid effect. Toxicol Appl Pharmacol 2000; 168: 244-252

Diwi RL, Chang HC, Doerge DR. Anti-thyroid isoflavones from soybean. Biochem Pharmacol 1997; 54: 1087-1096

Doerge DR & Takazawa . Mechanism of thyroid peroxidase inhibition by ethylenethiourea. Chem Res Toxicol 1990; 3: 98-101

Diwi RL, Doerge DR. Inhibition of thyroid peroxidase by dietary flavonoids. Chem Res Toxicol 1996; 9: 16-23

Doerge DR & Chang HC. Inactivation of thytoid peroxidase by soy isoflavones, in vitro and in vivo. J. Chromatogr. B 2002; 269-279

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Kurtoglu S, Özdemir A. Fetalneonatal hyperthyroidism: diagnostic and therapeutic approachment. Turk Pediatri Ars 2017; 52: 1-9

Neary JT. Soodak M, Maloof F. Iodination by thyroid peroxidase. Meth Enzymol 1984; 107: 445-475

Taurog A & Dorris ML. A re-examination of the proposed inactivation of thyroid peroxidase in the rat thyroid by propylthiouracil. Endocrinology 1989; 124: 3038-3042

Weisberger EK. Species differences in response to aromatic amines. In: Langenbach R, Nesnow S, Rice JM (Eds) Organ and Species Specifity in Chemical Carcinogenesis. Plenum Press , New York, 1983

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

The review manager did have consequent email exchange with the corresponding author before the TC. Even though a formally written answer was not provided at that moment, the information needed to prepare the TC was given. Moreover AOP42 authors also attended AOP54 TC from the beginning and had many relevant input to

Annex 4: Slides presented at the TC to support discussion

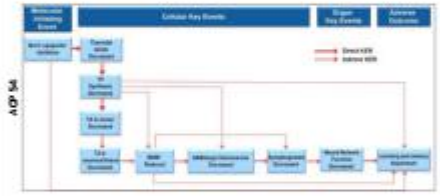
End of review TC
AOP 42 and 54

Overlapping issues and questions

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

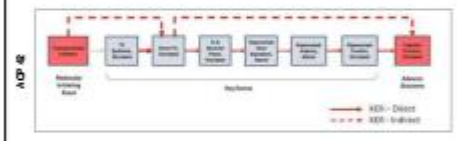
- Brief overview of both AOP 54 and 42 (Review manager)
- Comments on common key events: TH synthesis decreased, T4 in serum decreased, TH in neuronal tissue decreased
- How addressing AOPs sharing Key event(s) AND adverse outcome(s) with different intermediary key events?

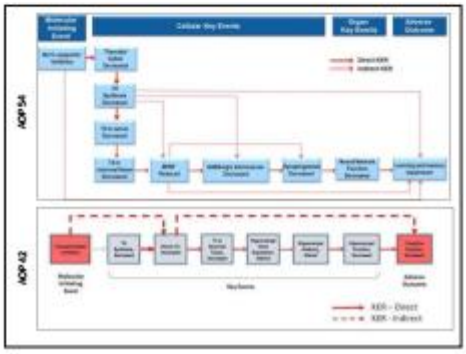
AOP 54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children

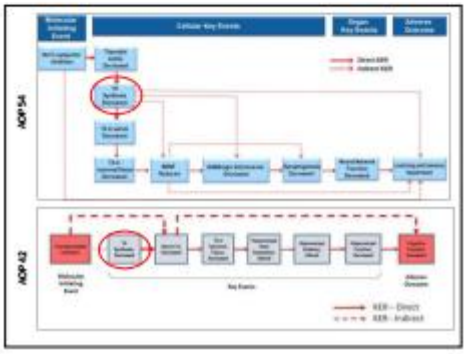


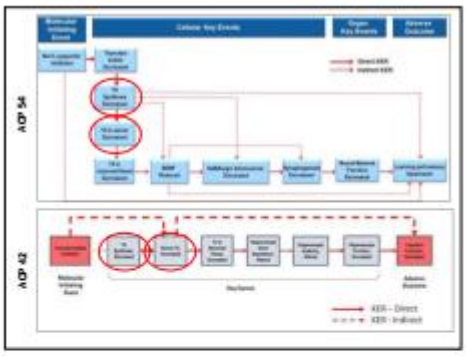
This AOP establishes the link between the inhibition of iodide/sodium symporter inhibition and the consequences on learning and memory deficits in children.

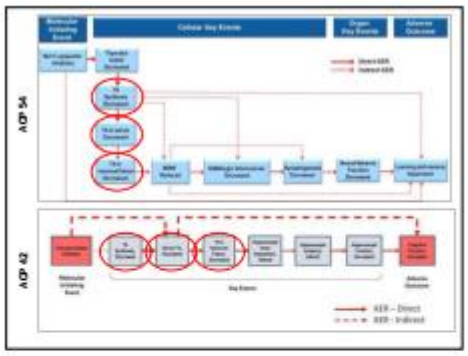
AOP 42: Inhibition of thyroperoxidase and subsequent Adverse Neurodevelopmental Outcomes in Mammals











- 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

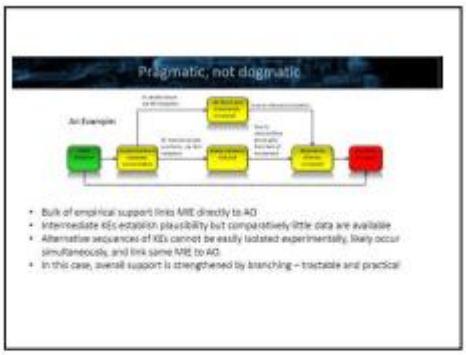
T4 in serum, decreased

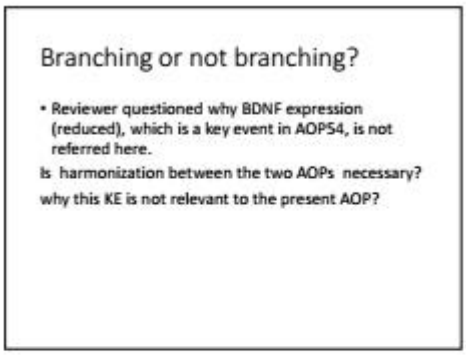
- Reviewers highlighted the need to recommend some methodologies for TH measurements as there are numerous (HPLC, MS, ELISA, RIA) with different sensitivities.
- A related point raised by two reviewers is the crucial need for standardized methodologies and ask authors to specify if one is preferred among 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.

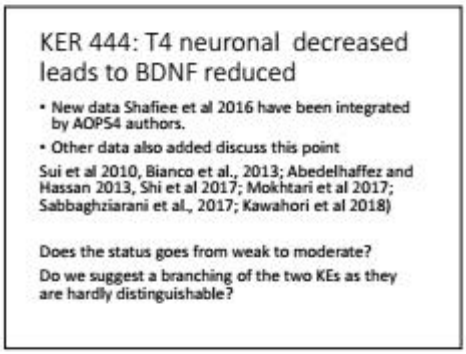
T4 in neuronal tissue, decreased

- TH levels being different within brain structures. Dissection method is crucial for reproducibility and should be clearly mentioned

Harmonization between AOPs?


Pragmatic, not dogmatic
An Example
Bulk of empirical support links MIE directly to AO
Intermediate KEs establish plausibility but comparatively little data are available
Alternative sequences of KEs cannot be easily isolated experimentally, likely occur simultaneously, and link same MIE to AO.
In this case, overall support is strengthened by branching – tractable and practical


Branching or not branching?
Reviewer questioned why BDNF expression (reduced), which is a key event in AOP54, is not referred here.
Is harmonization between the two AOPs necessary?
why this KE is not relevant to the present AOP?

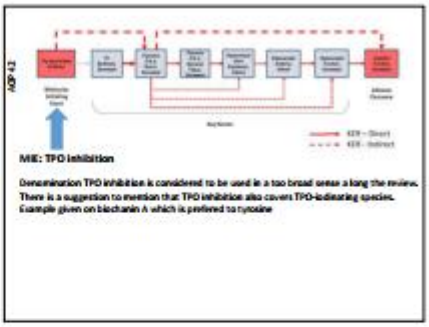

KER 444: T4 neuronal decreased leads to BDNF reduced
New data Shafiee et al 2016 have been integrated by AOP54 authors.
Other data also added discuss this point
Sui et al 2010, Bianco et al., 2013; Abedelhaffez and Hassan 2013, Shi et al 2017; Mokhtari et al 2017; Sabbaghziarani et al., 2017; Kawahori et al 2018)
Does the status goes from weak to moderate?
Do we suggest a branching of the two KEs as they are hardly distinguishable?


AOP 54


AOP 42


AOP 54

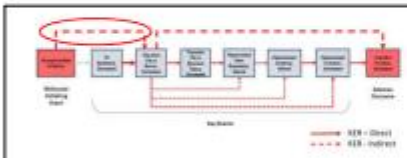
AOP 42 specific issues



Denomination TPO inhibition is considered to be used in a too broad sense along the review. There is a suggestion to mention that TPO inhibition also covers TPO-iodinating species. Example given on biochanin A which is preferred to tyrosine

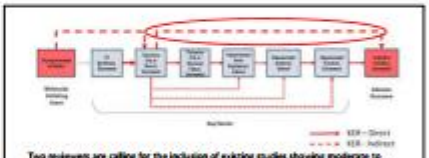
- Given that sensitivity to decreased levels of TH depends on moment of exposure and the storage capacity of iodine and thyroid hormones. Reviewers suggest to add in the KE description
  - Impact of Tg antibodies or age
  And to discuss in more details
  - factors influence iodine intake (age, diet ...) differential iodine storage capacities varies with life stages
  - reduction of thyroxine levels with genistein were not associated with cognitive impairment unless an iodine defect exists.
  - Direct fetal TPO inhibition and consequent brain/ hippocampal development in human should be mentioned (and the associated knowledge gap).

- *Hippocampal gene expression*
- RM3 agrees with the authors on the emphasis placed on the hippocampus given the most scientifically available literature but reminds that decreased cognitive function could have multiple origins.
- RM4 states that genome wide profiles or microarray studies are not specific enough to measure the effect of TH levels on gene expression. A prerequisite on hypothyroid hippocampus gene expression would be needed.



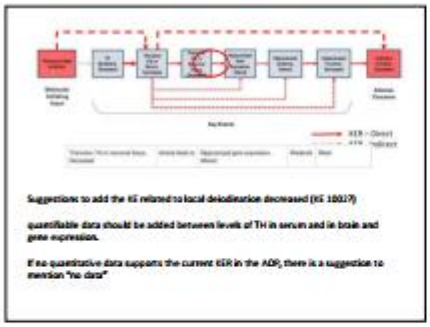
In Chang & Doerge 2008 and Doerge &Chang 2002, genistein administration was shown to lead to into a 80% inhibition of TPO activity but no effects were observed on TH levels in rat.

This should be added in uncertainties and inconsistencies.



Two reviewers are calling for the inclusion of existing studies showing moderate to severe decreases in circulating T_4 leading to minor or undetectable adverse effects on brain morphology in the offspring

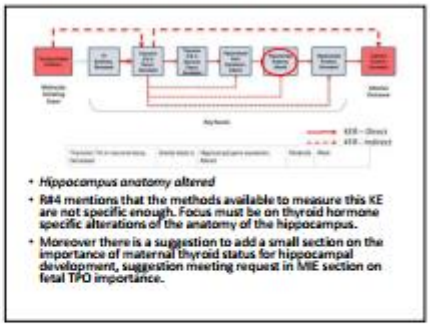
Quantitative relationship between the necessary decrease of thyroxine levels in dams, the resulting decrease of thyroxine in fetal brain and a neurodevelopmental adverse outcome is poorly understood and compounds with similar effects on thyroxine levels do not necessarily result in a comparable or in an adverse outcome at all (e.g., PTU versus ETU, Mancozeb Case Study, European Commission, 2017). The different outcomes for PTU vs ETU are currently not understood, whether iodothyronine deiodinase type I inhibition by PTU might play a role remains open (European Commission, 2017). Such a case should be included in the AOP and discussed.



Suggestions to add the KE related to local deiodination decreased (KE 18027)

quantifiable data should be added between levels of TH in serum and in brain and gene expression.

If no quantitative data supports the current KER in the AOP, there is a suggestion to mention "no data"



- *Hippocampus anatomy altered*
- R#4 mentions that the methods available to measure this KE are not specific enough. Focus must be on thyroid hormone specific alterations of the anatomy of the hippocampus.
- Moreover there is a suggestion to add a small section on the importance of maternal thyroid status for hippocampal development, suggestion meeting request in MIE section on fetal TPO importance.

5. AOPs are living documents

- AOPs are a way of organizing existing knowledge
- As methods for observing biology evolve:
  - New possibilities for KEs
  - Ability to measure KEs with greater precision/accuracy
- As new experiments are published:
  - Weight of evidence supporting (or rejecting) KERs grows
  - New AOPs and new branches in AOP networks discovered
- There is no objective "complete AOP"
  - There is only useful or not useful for a given application

Regulatory aspect

AOPs are modular

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

AOPs are living documents

- KE and KER descriptions can be expected to evolve over time
- As descriptions are updated and expanded – all AOP descriptions then link to update automatically

AOP networks for prediction

- Entry of structured information in KE allows for de facto assembly of AOP networks.

Regulatory applicabilities

As it currently stands, divergent opinions arise as to the applicability of the AOP for regulatory purposes.

Reviewer # 1 considers that the regulatory application can be quickly achieved, reviewers # 2 # 3 # 4 consider that a regulatory application is limited as long as quantifications are not achieved and standardized. Nevertheless, reviewers mention the possibility of direct applications for screening tests, either on neurotoxicity or for identification of endocrine disruptors.

Reviewer # 5 is more critical of the direct applicability at the regulatory level, the idea being that a percentage of TPO inhibition is needed to trigger the cascade of events described in AOP42 and that negative cases exist and should be mentioned (in reference to the genistein case).

What's next for AOP42

Next:

1) compilation of today's discussion and decisions in the final report
2) Revision is going to be 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