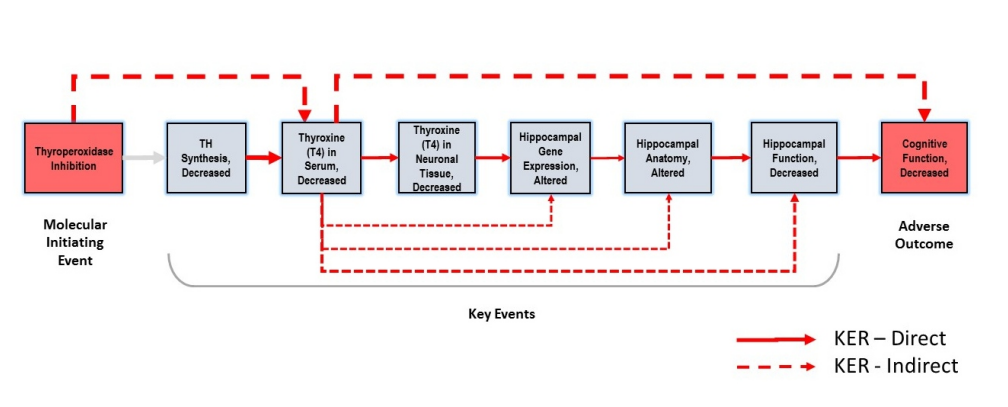
AOP ID and Title:

AOP 42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals
Short Title: TPO Inhibition and Altered Neurodevelopment

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	WPHA/WNT Endorsed	1.10	Included in OECD Work Plan

Abstract

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), results in decreased thyroid hormone (TH) synthesis, and subsequent reduction in circulating concentrations of THs. 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. Herein, we discuss the implications of developmental TPO inhibition for hippocampal anatomy, function, and ultimately neural function controlled by the hippocampus. The biochemistry of TPO and its essentiality for TH synthesis is well known across species. 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. 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 our 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.

Background

This AOP was originally started on the Chemical Mode of Action WIKI sponsored by WHO/IPCS. The MOA was originally described and published by Zoeller and Crofton (Crit Rev Toxicol 2005). Thanks to the following contributors whose work on the MOA-WIKI fostered further development on the AOP wiki: Michelle Embry, Richard Judson, Vicki Dellarco, Chihae Yang, Kevin Crofton.

Zoeller RT, Crofton KM. Mode of action: developmental thyroid hormone insufficiency--neurological abnormalities resulting from

exposure to propylthiouracil. Crit Rev Toxicol. 2005 Oct-Nov;35(8-9):771-81

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	279	Thyroperoxidase, Inhibition	Thyroperoxidase, Inhibition
2	KE	277	Thyroid hormone synthesis, Decreased	TH synthesis, Decreased
3	KE	281	Thyroxine (T4) in serum, Decreased	T4 in serum, Decreased
4	KE	280	Thyroxine (T4) in neuronal tissue, Decreased	T4 in neuronal tissue, Decreased
5	KE	756	Hippocampal gene expression, Altered	Hippocampal gene expression, Altered
6	KE	757	Hippocampal anatomy, Altered	Hippocampal anatomy, Altered
7	KE	758	Hippocampal Physiology, Altered	Hippocampal Physiology, Altered
8	AO	402	Cognitive Function, Decreased	Cognitive Function, Decreased

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Thyroperoxidase, Inhibition	adjacent	Thyroid hormone synthesis, Decreased	High	Low
Thyroid hormone synthesis, Decreased	adjacent	Thyroxine (T4) in serum, Decreased	High	Moderate
Thyroxine (T4) in serum, Decreased	adjacent	Thyroxine (T4) in neuronal tissue, Decreased	Moderate	Moderate
Thyroxine (T4) in neuronal tissue, Decreased	adjacent	Hippocampal gene expression, Altered	Moderate	Low
Hippocampal gene expression, Altered	adjacent	Hippocampal anatomy, Altered	Moderate	Low
Hippocampal anatomy, Altered	adjacent	Hippocampal Physiology, Altered	Moderate	Low
Hippocampal Physiology, Altered	adjacent	Cognitive Function, Decreased	High	Moderate
Thyroperoxidase, Inhibition	non-adjacent	Thyroxine (T4) in serum, Decreased	High	Moderate
Thyroxine (T4) in serum, Decreased	non-adjacent	Hippocampal gene expression, Altered	High	Low
Thyroxine (T4) in serum, Decreased	non-adjacent	Hippocampal anatomy, Altered	High	Low
Thyroxine (T4) in serum, Decreased	non-adjacent	Hippocampal Physiology, Altered	Moderate	Low
Thyroxine (T4) in serum, Decreased	non-adjacent	Cognitive Function, Decreased	High	Moderate

Stressors

Name	Evidence
Methimazole	High
Propylthiouracil	High

Overall Assessment of the AOP

Overall Assessment of the AOP

The following summary tables for:

1.Support for Biological Plausibility of KERS

2. Support for Essentiality of KEs

3. Empirical Support for KERs

Can be downloaded at:
https://aopwiki.org/system/dragonfly/production/2018/08/10/46w2o2kkl4_TPO_AOP_Summary_Tables_20180602.pdf

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
During brain development	High
Development	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Male	High
Female	High

- **Chemicals**: This AOP applies to a wide range of chemicals structures that inhibit TPO either in vivo or in vitro. Well recognized positive controls include propylthiouracil (PTU) and methimazole (MMI). There are 100s of other chemicals known to inhibit TPO in vitro (e.g., Paul-Friedman et al., 2016).
- **Sex**: This AOP applies to males and females. Disruption of thyroid hormone regulation during fetal and early postnatal develop, as well as the subsequent adverse impacts on nervous system development are similar in both sexes. There are no compelling data to suggest sex differences in susceptibility to TH disruption mediated by inhibition of TPO during development.
- **Life stages**: The relevant life stages for this AOP are fetal and early postnatal ages during critical windows of nervous system development where thyroid hormones guide normal development of the brain. There are clear windows of developmental susceptibility and different brain regions show distinct ontogenetic profiles for TH requirements. Distinct phenotypes have been described in both humans and animal models for different periods of TH insufficiency. The influence of maternal thyroid status prior to onset of fetal thyroid function is an important consideration. This AOP does not apply to adult life states.
- **Taxonomic**: Based on the majority of the available evidence the taxonomic applicability domains of this AOP is mammals. Most evidence for this AOP has been gathered primarily from laboratory rodents and humans. However, there are supporting data from amphibians and birds for TPO inhibition leading to altered TH profiles. Due to the conserved nature of TH synthesis, transport, metabolism and transcriptional activity, this AOP is likely to be applicable to other classes of vertebrates where thyroid hormones drive development of the nervous system (e.g., birds, fish, reptiles). However, species-specific differences in development, ADME (adsorption, distribution, metabolism, and elimination), compensatory endocrine responses may influence the outcomes, particularly from a quantitative standpoint.

Essentiality of the Key Events

It is widely accepted that each of the key events is essential.

- Molecular Initiating Event: The molecular initiating event, i.e. inhibition of TPO, is the essential event to initiate this AOP, as supported by in vitro and in vivo evidence. TPO is the only enzyme capable of de novo TH synthesis (Taurog, 2005). TPO is classically defined as a complex enzyme with multiple catalytic cycles capable of iodinating multiple species (Divi et al., 1997). However, in the context of this AOP we are using TPO inhibiton not in the classical sense, but instead to refer to the results derived from the assays commonly used assays to investigate environmental chemicals (e.g., guiacol oxidation). A number of studies have demonstrated that cessation of exposure is known to result in a return to normal levels of TH synthesis and circulatory hormone levels (Cooper et al., 1983). Many in vivo and in vitro studies consistently demonstrate enzyme inhibition with similar chemicals for multiple species (Taurog, 1999; Paul et al., 2013; Vickers et al., 2012).

- Thyroid hormone synthesis, Decreased. A number of studies have demonstrated a correlation between TPO activity and decreased TH synthesis (e.g., Vickers et al., 2012). Thyroid gland T4 concentrations, as well as serum TH, are decreased in response to thyroidectomy and recover when in-vitro derived follicles are grafted in athyroid mice (Antonica et al., 2012).
- Thyroxine (T4) in serum, Decreased. Inhibition of TPO is widely accepted as resulting in decreased TH synthesis in the thyroid gland, which results in decreased serum T4 concentration (Taurog, 2005). Stop/recovery experiments demonstrate recovery of serum thyroxine concentrations due to cessation of developmental exposure to chemical stressors (e.g., Crofton et al., 2000), with similar findings in adult rats (Cooper et al.,1984). Studies in adult animals show a similar recovery after cessation of dosing (e.g., Hill et al., 1998).
- Thyroxine (T4) in neuronal tissue, decreased: Mulitple studies have demonstrated that fetal brain TH levels, previously decreased by maternal exposure to TPO inhibitors or thyroidectomy, recovered following maternal dosing with T4 (e.g., Calvo et al., 1990). In addition, upregulation of deiodinase has been shown compensate for some loss of neuronal T3 (Escobar-Morreale et al., 1997). Indirect evidence shows that T4 replacement that bring circulating T4 concentration back to normal, leads to recovery of brain TH and prevents downstream effects including alterations in gene expression in the developing brain.
- Hippocampal Gene Expression, Altered: It is well established specific genomic pathways underlie the progression of a number of neurodevelopmental processes in the hippocampus. There is some evidence from ex vivo studies that administration of growth factors will reverse the hippocampal dysplasia seen in Jacob/Nsfm knockout mice (Spilker et al., 2016). Less is known about the impact of hormone replacement on TH-responsive gene expression and the qualitative and quantitative relationships between altered TH-dependent gene expression in this brain region and altered hippocampal cytoarchitectural anatomy.
- Hippocampal anatomy, altered: It is well accepted that normal hippocampal anatomy is critical for hippocampal physiological function, and that alterations in anatomy lead to altered neuronal activity in the hippocampus (Lee et al., 2015; Grant et al., 1992; Spilker et al., 2016).
- Hippocampal physiology, altered: It is a well-accepted assertion that hippocampal synaptic integrity and neuronal plasticity are essential for spatial information processing in animals and spatial and episodic memory in humans. However, other brain regions also can influence these complex behaviors. Limited data from studies in BDNF knockout animals demonstrate that deficits in hippocampal synaptic transmission and plasticity, and downstream behaviors can be rescued with recombinant BDNF (Aarse et al., 2016; Andero et al., 2014).
- Cognitive function, decreased: It is a well-known fact that TH are critical for normal nervous system development (Williams et al., 2008). And this includes development of the hippocampus which plays a major role in spatial, temporal, and contextual memory. Indeed, most developed countries check for childhood hypothyroidism at birth to immediately begin replacement therapy. This has been shown to alleviate most adverse impacts of hypothyroidism in congenitally hypothyroid children (Derksen-Lubsen and Verkerk 1996; Zoeller and Rovet, 2004). The essentiality of the relationship between decreased TH levels and this adverse outcome is well accepted. Decreased cognitive function specific to the hippocampal region are particularly associated with decrements in memory and learning domains of cognition.

Weight of Evidence Summary

Biological plausibility: Biological plausibility refers to the structural or functional relationship between the key events based on our fundamental understanding of "normal biology". In general, the biological plausibility and coherence linking TPO inhibition through decreases in circulating concentrations of THs, to adverse impacts in the developing hippocampus and subsequent cognitive behaviors is very solid. That thyroidal TPO is the sole enzyme capable of de novo TH synthesis and the only source of circulating T4, is beyond doubt. It is also widely accepted that circulating T4 is the only source of nervous system T4 that is converted to the biological active T3. The direct link between reduced brain TH concentrations and reduced expression of TR regulated genes is supported by a plethora of literature. However, the direct connection between exactly which genes are regulated and at which developmental periods is not as clear. Similarly, the precise relationships between gene expression and hippocampal anatomy is not completely known. A lot of the work in this area has been done for a limited number of genes and specific hippocampal anatomical anomalies that are known to alter both the physiological and function of the hippocampus, and subsequent cognitive function. That said, it is widely acknowledged that abnormal TH levels during fetal and early development lead to adverse hippocampally-driven cognitive function in humans and laboratory animals.

1. The biochemistry of TPO and its essentiality for TH synthesis is well known across species, with the evidence across vertebrate species, including amphibians, birds, rodents, pigs, and humans.
2. The relationship between TH synthesis and serum TH concentrations is well accepted scientific dogma. There are no other pathways in mammals that will maintain homeostatic serum TH concentrations.
3. Serum is the only source of thyroxine for the brain. In the brain, deiodinases convert T4 to T3, the more biologically active moiety. Some serum T3 may also contribute to total brain T3. These are well accepted scientific facts.
4. It is well established that T3 binding to thyroid receptors controls critical transcriptional and translational processes in the developing brain, including the hippocampus. Lack of TH results in abnormal development of the structure and physiological function in the hippocampus. What is not well known is exactly which genes, at what fetal and postnatal ages, are responsible for the development of the complexity of hippocampal anatomy and function.
5. Lastly, the biological plausibility that changes in brain structure and physiology, and specifically aberrations in the hippocampus, lead to abnormal cognitive function is well accepted.

Concordance of dose-response relationships:

There are a large number of studies that include correlative evidence between exposure to TPO inhibitors and downstream KEs, as well as the AO. In addition, there are also studies with dose-response relationships that indirectly link KEs, especially from serum TH concentrations to downstream KEs and the AO. There is a more limited set of studies in which two directly linked key events were considered in the same study following exposure to TPO inhibitors or other stressors (e.g., thyroidectomy, gene knockouts). These later studies, while providing critical data for causatively linking the key events, provide less information on the concordance of the dose-response relationship, especially for the latter KEs. For earlier KEs, Zoeller and Crofton (2005) provide good dose response concordance for data derived from the TPO inhibitor PTU. While limited in number, in general these studies provide moderate confidence that downstream key events occurred at concentrations equal to or greater than those directly upstream. In addition, there are several quantitiative models that, based on empirical data, can predict dose relationships between many of the early KEs up to and including serum hormone concentrations (e.g., Degon et al., 2008; Fisher et al 2013; Ekerot et al., 2012;

Leonard et al., 2016). A more recent model predicts neuroanatomical anomalies based on serum and brain T4 concentrations (Hassan et al., 2017).

All this information taken together, provide strong concordance of the dose-relationships for all KEs.

Temporal concordance among the key events and adverse effect: There are two aspects of the temporal concordance of the key events in a developmental AOP. The first is the temporal concordance refers to the degree to which the data support the hypothesized sequence of the key events; i.e., the effect on KE1 is observed before the effect on KE2, which is observed before the effect on KE3, and so on. This translates to the temporal concordance of the AOP from TPO inhibition to decreased TH synthesis, reduced circulating TH concentrations, decreased nervous system TH, altered gene expression and anatomy in the hippocampus, and subsequent alterations in hippocampal physiology that result in decrements in cognition. The strength of the temporal concordance between these KEs varies from weak to strong (see Appendix Tables and individual KEs for detailed information). There is strong evidence for the early direct KEs from both empirical and modeling studies, and for many of the later KEs via the indirect KERs. The temporal concordance between TPO inhibition and TH synthesis is clearly evidenced by data from ex vivo and in vitro studies, as well as computational models (Leonard et al., 2016; Degon et al., 2008; Zoeller and Crofton, 2005; Cooper et al., 1983; Goldey et al. 1985; Christenson et al 1995). Data supporting the temporal concordance for the later KEs, i.e., from serum TH to changes in hippocampal physiology are limited or lacking.

The second aspect of temporal concordance for developmental AOPs is evidenced by demonstrations for critical windows of development where key events are perturbed, for which the effects are permanent and found during early development and throughout adulthood (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of serum THs that result in subsequent alterations in all downstream KEs including the AO cognitive function later in development and adulthood. Indeed, the literature is replete with studies that demonstrate critical windows of susceptibity to thyroid disruption and adverse impacts on the developing brain. For reviews see: Morreale de Escobar (2001); Howdshell (2002). There are also many studies in which downstream direct and indirect consequences of TPO inhibition and other stressors (e.g., iodine deficiency, thyroidectomy, gene knockouts) have been ameliorated by administration of thyroxine. For example, based on the indirect link between serum TH hormone concentrations and decrements in hippocampally-mediated spatial behaviors, it commonly accepted dogma that there are critical windows of development in which exposure and hormone reduction lead to permanent effect on cognitive functions. Indeed, most developed countries have mandatory screening for congenital hypothyroidism, so that hormone replacement therapy can begin immediately, and thus prevent declines in IQ in childhood. (e.g., the temporal concordance between the MIE, KEs and AO. Overall, all available data are consistent with the temporal concordance of this AOP.

Consistency: There is no data that we are aware of that does not support the pattern of key events described in this AOP. A limited number of studies with measurements of directly linked KEs within the same study, the fact that the majority of the data was generated with single-stressor studies (e.g., one chemical dose, knockout, or thyroidectomy), coupled with likely differences in sensitivity of many of the measured endpoints (e.g., gene expression), make it difficult to determine quantitative consistency between studies. Nonetheless, the occurrence of the final AO, when upstream key events are observed is extremely consistent. It is also very important to note that the AO, alterations in cognitive function, is not likely to be specific solely to this AOP. Many of the key events included in this AOP overlap with AOPs linking other molecular initiating events to alterations in hippocampally-driven cognitive behaviors such as spatial learning in rats and IQ in humans.

Uncertainties, inconsistencies, and data gaps:

There are several areas of uncertainty and data gaps in the current AOP:

- There is a lack of quantitative information for several the KERs. These gaps hamper development of quantitative models that will allow linkages between the MIE and AO. Quantitative models are needed to facilitate efficient use of data on ~1000 chemicals from in vitro TPO assays (e.g., Paul-Friedman et al., 2016) to predict potential adverse outcomes. Computational models are needed to describe relationships between serum and brain TH as a critical KER. With an additional metric of TH action in brain, this may be sufficient for application to computational prediction in the regulatory arena. These gaps include:
  - Insufficient information exists to quantitatively link the degree of in vivo TPO inhibition required to elicit specific decrements in circulating T4 concentrations; Genistein is an example of where a very large degree of inhibition may be required to have an impact on serum TH;
  - There is a lack of data to quantitatively associate serum TH concentrations with TH concentrations in specific brain regions;
  - Presently TH-responsive gene expression in hippocampus has not been quantitatively linked to changes in hippocampal anatomy, hippocampal function, and subsequent adverse cognitive effects. Neither has this AOP considered the nongenomic actions of TH on cell signaling in brain.
- There is limited available data that inform a quantitative relationship between in vitro and in vivo inhibition of TPO (but see Vickers et al., 2012).
- Compensatory feedback systems are not included in this AOP. For example, it is well known that with chemicals that inhibit TPO (e.g., PTU) decrease circulating TH concentrations which activates the hypothalamic-pituitary feedback system (Capen, 1997). This leads to increased secretion of TSH, which upregulates TH synthesis in the thyroid gland (e.g., McCain, 1995; Capen, 1997; Hill et al., 1998). There is also compensation within the developing nervous system where low tissue T4 concentrations upregulates deiodinases in an attempt to maintain proper levels of T3 (e.g., Morse et al., 1996; Sharlin et al 2010). These and other compensatory systems are likely to be differentially active across different developmental ages and in different brain regions
- Lastly, there is some uncertainty in the literature about the role of thyroid stimulating hormone (TSH) in thyroid hormone based adverse outcome pathways and the relevance of rodent data for humans. It is clear that TSH is a key event in the AOP for rat thyroid follicular tumors (McCain, 1995; Hill et al., 1998) and this pathway is not deemed relevant to humans (Axelrad et

al., 2005). However, it is critically important to note that the current AOP does not contain TSH as a KE. This is because, while TSH may play a role in feedback-driven compensatory processes to maintain peripheral hormone concentrations, it is not directly involved in brain development. In this AOP, TSH may be used as a supporting biomarker for alterations in circulating THs, however, it is not a perfect surrogate. There are also numerous examples of pharmaceutical and industrial chemicals that alter circulating THs in rats without any measurable change in TSH (NTP, 1990; O'Connor et al., 1998 2000; Liu et al., 1994; Zoeller et al., 2005; Morse et al., 1996; Goldey et al., 1995; Lau et al 2003; Schneider et al., 2011). In the absence of TSH changes, exposure to some of these chemicals do result in adverse neurological outcomes (e.g., Goldey and Crofton, 1998; Crofton, 2004; Zoeller et al., 2005; Cope et al., 2015). Therefore, stressor-induced changes in TH, not in TSH, are responsible for adverse neurological outcomes.

Quantitative Consideration

Assessment of quantitative understanding of the AOP: Currently, there are quantitative models for the early KERs from TPO inhibiton to serum hormone concentrations, but none for later KERs. And only one of these models the KERs during early development (Fisher et al., 2013). A recent study by Hassan et al. (2017) quantitatively linked PTU-induced TH synthesis declines in the dam and the fetus to decrements in serum and brain TH concentrations to a structural malformation in the postnatal brain. In this study, estimates of TPO inhibition were derived from glandular and serum PTU and TH concentrations. For the rest of the KERs in this AOP, there is a varying amount of data from dose-response studies that demonstrate increasing impact with increasing chemical dose for all the KEs, and the direct and indirect KERs. At present, the overall quantitative understanding of the AOP is insufficient to directly link a measure of chemical potency as a TPO inhibitor to a quantitative prediction of effect on cognitive function (e.g., IQ in humans, learning deficits in rodents). Empirical information on dose-response relationships for the intermediate KEs, currently unavailable, would inform a computational, predictive model for thyroid disruption via TPO inhibition.

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Appendix 1

List of MIEs in this AOP

Event: 279: Thyroperoxidase, Inhibition

Short Name: Thyroperoxidase, Inhibition

Key Event Component

Process	Object	Action
iodide peroxidase activity	thyroid peroxidase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	MolecularInitiatingEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	MolecularInitiatingEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	MolecularInitiatingEvent
Aop:271 - Inhibition of thyroid peroxidase leading to impaired fertility in fish	MolecularInitiatingEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	MolecularInitiatingEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	MolecularInitiatingEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	MolecularInitiatingEvent

Stressors

Name
2(3H)-Benzothiazolethione
2-mercaptobenzothiazole
Ethylene thiourea
Mercaptobenzothiazole

Name
Methimazole
Propylthiouracil
Resorcinol
Thiouracil
Ethylenethiourea
Amitrole
131-55-5
2,2',4,4'-Tetrahydroxybenzophenone
Daidzein
Genistein
4-Nonylphenol
4-propoxyphenol
Sulfamethazine

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
thyroid follicular cell

Organ term

Organ term
thyroid follicle

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

There is a wealth of information on the inhibition of TPO by drugs such as MMI and PTU, as well as environmental xenobiotics. In the landmark paper on TH system disruption by environmental chemicals, Brucker-Davis (1998) identified environmental chemicals that depressed TH synthesis by inhibiting TPO. Hurley (1998) listed TPO as a major target for thyroid tumor inducing pesticides. More recent work has tested over 1000 chemicals using a high-throughput screening assay (Paul-Friedman et al., 2016).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
humans	Homo sapiens	High	NCBI
pigs	Sus scrofa	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
chicken	Gallus gallus	High	NCBI
zebrafish	Danio rerio	High	NCBI

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	High	NCBI
mouse	Mus musculus		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Taxonomic:

This KE is plausibly applicable across vertebrates. TPO inhibition is a MIE conserved across taxa, with supporting data from experimental models and human clinical testing. This conservation is likely a function of the high degree of protein sequence similarity in the catalytic domain of mammalian peroxidases (Taurog, 1999). Ample data available for human, rat, and porcine TPO inhibition demonstrate qualitative concordance across these species (Schmultzer et al., 2007; Paul et al., 2013; Hornung et al., 2010). A comparison of rat TPO and pig TPO, bovine lactoperoxidase, and human TPO inhibition by genistein demonstrated good qualitative and quantitative (40–66%) inhibition across species, as indicated by quantification of MIT and DIT production (Doerge and Chang, 2002). Ealey et al. (1984) demonstrated peroxidase activity in guinea pig thyroid tissue using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate that is oxidized by the peroxidase to form a brown insoluble reaction product. Formation of this reaction product was inhibited by 3-amino-1,2,4-triazole and the TPO inhibitor, methimazole (MMI). A comparative analysis of this action of MMI between rat- and human-derived TPO indicates concordance of qualitative response. Data also suggest an increased quantitative sensitivity to MMI in rats compared to humans (Vickers et al., 2012). Paul et al. (2013) tested 12 chemicals using the guaiacol assay using both porcine and rat thyroid microsomes. The authors concluded that there was an excellent qualitative concordance between rat and porcine TPO inhibition, as all chemicals that inhibited TPO in porcine thyroid microsomes also inhibited TPO in rat thyroid microsomes when tested within the same concentration range. In addition, these authors noted a qualitative concordance that ranged from 1.5 to 50-fold differences estimated by relative potency. Similarly, Takayama et al. (1986) found a very large species difference in potency for sulfamonomethoxine between cynomologus monkeys and rats.

Life stage:

Applicability to certain life stages may depend on the species and their dependence on maternally transferred THs during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf and not at 24 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TPO inhibition.

Sex:

This KE is plausibly applicable to both sexes. The molecular components responsible for TH synthesis, including TPO, are identical in both sexes. Therefore inhibition of TPO is not expected to be sex-specific.

Key Event Description

Thyroperoxidase (TPO) is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as the enzymatic catalyst for thyroid hormone (TH) synthesis. TPO catalyzes several reactions in the thyroid gland, including: the oxidation of iodide; nonspecific iodination of tyrosyl residues of thyroglobulin (Tg); and the coupling of iodotyrosyls to produce Tg-bound monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Divi et al., 1997; Kessler et al., 2008; Ruf et al., 2006; Taurog et al., 1996). The outcome of TPO inhibition is decreased synthesis of thyroxine (T4) and triiodothyronine (T3), a decrease in release of these hormones from the gland into circulation, and unless compensated, a consequent decrease in systemic concentrations of T4, and possibly T3. The primary product of TPO-catalyzed TH synthesis is T4 (Taurog et al., 1996; Zoeller et al., 2007) that would be peripherally or centrally deiodinated to T3.

It is important to note that TPO is a complex enzyme that has two catalytic cycles and is capable of iodinating multiple species (Divi et al., 1997). Alterations in all of these events are not 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 to investigate environmental chemicals.

Figure 1 illustrates the enzymatic and nonenzymatic reactions mediated by TPO that result in the synthesis of thyroxine (T4) .

Inhibition of TPO can be reversible, with transient interaction between the enzyme and the chemical, or irreversible, whereby suicide substrates permanently inactivate the enzyme. Reversible and irreversible TPO inhibition may be determined by the chemical structure, may be concentration dependent, or may be influenced by other conditions, including the availability of iodine (Doerge and Chang, 2002).

The ontogeny of TPO has been determined using both direct and indirect evidence in **mammals**. Available evidence suggests the 11th to 12th fetal week as the beginning of functional TPO in humans. In rodents, TPO function begins late in the second fetal week, with the first evidence of T4 secretion on gestational day 17 (Remy et al., 1980). Thyroid-specific genes appear in the thyroid gland according to a specific temporal pattern; thyroglobulin (*Tg*), TPO (*Tpo*), and TSH receptor (*Tshr*) genes are expressed by gestational day 14 in rats, and the sodium iodide symporter, NIS (*Nis*), is expressed by gestational day 16 in rats. Maturation to adult function is thought to occur within a few weeks after parturition in rats and mice, and within the first few months in neonatal humans (Santisteban and Bernal, 2005). Tg is first detected in human fetuses starting at 5th week of gestation and rises throughout gestation (Thorpe-Beeston et al., 1992), but iodine trapping and T4 production does not occur until around 10-12 weeks. Also, the dimerization of Tg, a characteristic of adult TH storage, is not found until much later in human gestation (Pintar, 2000). In rats, Tg immunoreactivity does not appear until day 15 of gestation (Fukiishi et al., 1982; Brown et al., 2000). The vast majority of research and knowledge on Tg is from mammals, although genomic orthologs are known for a variety of other species (Holzer et al., 2016). It is important to note that prior to the onset of fetal thyroid function, THs are still required by the developing fetus which until that time relies solely on maternal sources. Chemical-induced TPO inhibition can affect synthesis in the maternal gland and in the fetal gland.

The components of the TH system responsible for TH synthesis are highly conserved across vertebrates. In fish and amphibians TPO and NIS inhibition result in an expected decrease of TH synthesis (Hornung et al., 2010; Tietge et al., 2013; Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020) like in mammals. Although the TH system is highly conserved across vertebrates, there are some taxon-specific considerations.

Zebrafish and fathead minnows are oviparous **fish** species in which maternal THs are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic TH synthesis is initiated. Maternal transfer of THs to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

Inhibition of TPO can only occur after activation of embryonic TH synthesis mediated by TPO. Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for TPO is maternally transferred in relatively high amounts with subsequent mRNA degradation followed by initiation of embryonic transcription around hatching (Vergauwen et al., 2018).

How it is Measured or Detected

There are no approved OECD or EPA guideline study protocols for measurement of TPO inhibition. However, there is an OECD scoping document on identification of chemicals that modulate TH signaling that provides details on a TPO assay (OECD, 2017).

From the early 1960's, microsomal fractions prepared from porcine thyroid glands and isolated porcine follicles were used as a source of TPO for inhibition experiments (Taurog, 2005). Microsomes from human goiter samples (Vickers et al., 2012) and rat thyroid glands (Paul et al., 2013; 2014; Paul-Friedman et al., 2016) have also been used as a source of TPO.

TPO activity has been measured for decades via indirect assessment by kinetic measurement of the oxidation of guaiacol (Chang & Doerge 2000; Hornung et al., 2010; Schmutzler et al., 2007). This method is a low-throughput assay due to the very rapid kinetics of the guaiacol oxidation reaction. More recently, higher-throughput methods using commercial fluorescent and luminescent substrates with rodent, porcine, and human microsomal TPO have been developed (Vickers et al., 2012; Paul et al., 2013; 2014; Kaczur et al., 1997). This assay substitutes a pre-fluorescent substrate (Amplex UltraRed) for guaiacol, that when incubated with a source of peroxidase and excess hydrogen peroxidase, results in a stable fluorescent product proportional to TPO activity (Vickers et al., 2012). The stability of the fluorescent reaction product allows this assay to be used in a higher throughput format (Paul-Friedman et al., 2016). This approach is appropriate for high-throughput screening but does not elucidate the specific mechanism by which a chemical may inhibit TPO (Paul-Friedman et al., 2016), and as with most *in vitro* assays, is subject to various sources of assay interference (Thorne et al., 2010).

HPLC has been used to measure the activity of TPO via formation of the precursors monoiodotyrosine (MIT), diiodotyrosine (DIT), and both T3 and T4, in a reaction mixture containing TPO, or a surrogate enzyme such as lactoperoxidase (Divi & Doerge 1994). The tools and reagents for this method are all available. However, HPLC or other analytical chemistry techniques make this a low throughput assay, depending on the level of automation. A primary advantage of this *in vitro* method is that it directly informs hypotheses regarding the specific mechanism by which a chemical may impact TH synthesis in vitro.

In fish, increases of TPO mRNA levels are often used as indirect evidence of TPO inhibition in *in vivo*experiments (Baumann et al., 2016; Nelson et al., 2016; Wang et al., 2020).

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List of Key Events in the AOP

Event: 277: Thyroid hormone synthesis, Decreased

Short Name: TH synthesis, Decreased

Key Event Component

Process	Object	Action
thyroid hormone generation	thyroid hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:128 - Kidney dysfunction by decreased thyroid hormone	MolecularInitiatingEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:192 - Pendrin inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:193 - Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:271 - Inhibition of thyroid peroxidase leading to impaired fertility in fish	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	KeyEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	KeyEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term

thyroid follicular cell

Organ term

Organ term
thyroid gland

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-proylthiouracil is a common positive control for inhibition of TPO

Methimazole

Methimazole is a very common positve control for inhibition of TPO

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
Sus scrofa	Sus scrofa	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: This KE is plausibly applicable across vertebrates. Decreased TH synthesis resulting from TPO or NIS inhibition is conserved across vertebrate taxa, with *in vivo* evidence from humans, rats, amphibians, some fish species, and birds, and *in vitro* evidence from rat and porcine microsomes. Indeed, TPO and NIS mutations result in congenital hypothyroidism in humans (Bakker et al., 2000; Spitzweg and Morris, 2010), demonstrating the essentiality of TPO and NIS function toward maintaining euthyroid status. Though decreased serum T4 is used as a surrogate measure to indicate chemical-mediated decreases in TH synthesis, clinical and veterinary management of hyperthyroidism and Grave's disease using propylthiouracil and methimazole, known to decrease TH synthesis, indicates strong evidence for chemical inhibition of TPO (Zoeller and Crofton, 2005).

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred THs during the earliest phases of development. The earliest life stages of teleost fish (e.g., fathead minnow, zebrafish) rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). In externally developing fish species, decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. Therefore, it is still uncertain when exactly embryonic TH synthesis is activated and thus when exactly this process becomes sensitive to disruption. In fathead minnows, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It currently remains unclear when exactly embryonic TH production is initiated in zebrafish.

Sex: The KE is plausibly applicable to both sexes. THs are essential in both sexes and the components of the HPT-axis are

identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of TH levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in TH levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Description

The thyroid hormones (TH), triiodothyronine (T3) and thyroxine (T4) are thyrosine-based hormones. Synthesis of THs is regulated by thyroid-stimulating hormone (TSH) binding to its receptor and thyroidal availability of iodine via the sodium iodide symporter (NIS). Other proteins contributing to TH production in the thyroid gland, including thyroperoxidase (TPO), dual oxidase enzymes (DUOX), and pendrin are also necessary for iodothyronine production (Zoeller et al., 2007).

The production of THs in the thyroid gland and resulting serum concentrations are controlled by a negatively regulated feedback mechanism. Decreased T4 and T3 serum concentrations activates the hypothalamus-pituitary-thyroid (HPT) axis which upregulates thyroid-stimulating hormone (TSH) that acts to increase production of additional THs (Zoeller and Tan, 2007). This regulatory system includes: 1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH); 2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary; 3) hormonal transport by the plasma binding proteins; 4) cellular uptake mechanisms at the tissue level; 5) intracellular control of TH concentrations by deiodinating mechanisms; 6) transcriptional function of the nuclear TH receptor; and 7) in the fetus, the transplacental passage of T4 and T3 (Zoeller et al., 2007).

TRH and the TSH primarily regulate the production of T4, often considered a “pro-hormone,” and to a lesser extent of T3, the transcriptionally active TH. Most of the hormone released from the thyroid gland into circulation is in the form of T4, while peripheral deiodination of T4 is responsible for the majority of circulating T3. Outer ring deiodination of T4 to T3 is catalyzed by the deiodinases 1 and 2 (DIO1 and DIO2), with DIO1 expressed mainly in liver and kidney, and DIO2 expressed in several tissues including the brain (Bianco et al., 2006). Conversion of T4 to T3 takes place mainly in the liver and kidney, but also in other target organs such as in the brain, the anterior pituitary, brown adipose tissue, thyroid and skeletal muscle (Gereben et al., 2008; Larsen, 2009).

In **mammals**, most evidence for the ontogeny of TH synthesis comes from measurements of serum hormone concentrations. And, importantly, the impact of xenobiotics on fetal hormones must include the influence of the maternal compartment since a majority of fetal THs are derived from maternal blood early in fetal life, with a transition during mid-late gestation to fetal production of THs that is still supplemented by maternal THs. In humans, THs can be found in the fetus as early as gestational weeks 10-12, and concentrations rise continuously until birth. At term, fetal T4 is similar to maternal levels, but T3 remains 2-3 fold lower than maternal levels. In rats, THs can be detected in the fetus as early as the second gestational week, but fetal synthesis does not start until gestational day 17 with birth at gestational day 22-23. Maternal THs continue to supplement fetal production until parturition (see Howdeshell, 2002; Santisteban and Bernal, 2005 for review). The ontogeny of TPO inhibition during development by environmental chemicals represents a data gap.

Decreased TH synthesis in the thyroid gland may result from several possible molecular-initiating events (MIEs) including: 1) Disruption of key catalytic enzymes or cofactors needed for TH synthesis, including TPO, NIS, or dietary iodine insufficiency. Theoretically, decreased synthesis of Tg could also affect TH production (Kessler et al., 2008; Yi et al., 1997). Mutations in genes that encode requisite proteins in the thyroid may also lead to impaired TH synthesis, including mutations in pendrin associated with Pendred Syndrome (Dossena et al., 2011), mutations in TPO and Tg (Huang and Jap 2015), and mutations in NIS (Spitzweg and Morris, 2010). 2) Decreased TH synthesis in cases of clinical hypothyroidism may be due to Hashimoto's thyroiditis or other forms of thyroiditis, or physical destruction of the thyroid gland as in radioablation or surgical treatment of thyroid lymphoma. 3) It is possible that TH synthesis may also be reduced subsequent to disruption of the negative feedback mechanism governing TH homeostasis, e.g. pituitary gland dysfunction may result in a decreased TSH signal with concomitant T3 and T4 decreases. 4) More rarely, hypothalamic dysfunction can result in decreased TH synthesis.

Increased fetal TH levels are also possible. Maternal Graves disease, which results in fetal thyrotoxicosis (hyperthyroidism and increased serum T4 levels), has been successfully treated by maternal administration of TPO inhibitors (c.f., Sato et al., 2014).

It should be noted that different species and different life stages store different amounts of TH precursors and iodine within the thyroid gland. Thus, decreased TH synthesis via transient iodine insufficiency or inhibition of TPO may not affect TH release from the thyroid gland until depletion of stored iodinated Tg. Adult humans may store sufficient Tg-DIT residues to serve for several months to a year of TH demand (Greer et al., 2002; Zoeller, 2004). Neonates and infants have a much more limited supply of less than a week.

While the TH system is highly conserved across vertebrates, there are some taxon-specific considerations.

Zebrafish and fathead minnows are oviparous **fish** species in which maternal THs are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic TH synthesis is initiated. Maternal transfer of THs to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

Decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. The components of the TH system responsible

for TH synthesis are highly conserved across vertebrates and therefore interference with the same molecular targets compared to mammals can lead to decreased TH synthesis (TPO, NIS, etc.) in fish. Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for these genes is also maternally transferred and increasing expression of most transcripts during hatching and embryo-larval transition indicates a fully functional HPT axis in larvae (Vergauwen et al., 2018). Although the HPT axis is highly conserved, there are some differences between fish and mammals (Blanton and Specker, 2007; Deal and Volkoff, 2020). For example, in fish, corticotropin releasing hormone (CRH) often plays a more important role in regulating thyrotropin (TSH) secretion by the pituitary and thus TH synthesis compared to TSH-releasing hormone (TRH). Also, in most fish species thyroid follicles are more diffusely located in the pharyngeal region rather than encapsulated in a gland.

How it is Measured or Detected

Decreased TH synthesis is often implied by measurement of TPO and NIS inhibition measured clinically and in laboratory models as these enzymes are essential for TH synthesis. Rarely is decreased TH synthesis measured directly, but rather the impact of chemicals on the quantity of T4 produced in the thyroid gland, or the amount of T4 present in serum is used as a marker of decreased T4 release from the thyroid gland (e.g., Romaldini et al., 1988). Methods used to assess TH synthesis include, incorporation of radiolabeled tracer compounds, radioimmunoassay, ELISA, and analytical detection.

Recently, amphibian thyroid explant cultures have been used to demonstrate direct effects of chemicals on TH synthesis, as this model contains all necessary synthesis enzymes including TPO and NIS (Hornung et al., 2010). For this work THs was measured by HPLC/ICP-mass spectometry. Decreased TH synthesis and release, using T4 release as the endpoint, has been shown for thiouracil antihyperthyroidism drugs including MMI, PTU, and the NIS inhibitor perchlorate (Hornung et al., 2010).

Techniques for *in vivo* analysis of TH system disruption among other drug-related effects in fish were reviewed by Raldua and Piña (2014). TIQDT (Thyroxine-immunofluorescence quantitative disruption test) is a method that provides an immunofluorescent based estimate of thyroxine in the gland of zebrafish (Raldua and Babin, 2009; Thienpont et al., 2011; Jomaa et al., 2014; Rehberger et al., 2018). Thienpont used this method with ~25 xenobiotics (e.g., amitrole, perchlorate, methimazole, PTU, DDT, PCBs). The method detected changes for all chemicals known to directly impact TH synthesis in the thyroid gland (e.g., NIS and TPO inhibitors), but not those that upregulate hepatic catabolism of T4. Rehberger et al. (2018) updated the method to enable simultaneous semi-quantitative visualization of intrafollicular T3 and T4 levels. Most often, whole body TH level measurements in fish early life stages are used as indirect evidence of decreased TH synthesis (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020). Analytical determination of TH levels by LC-MS is becoming increasingly available (Hornung et al., 2015).

More recently, transgenic zebrafish with fluorescent thyroid follicles are being used to visualize the compensatory proliferation of the thyroid follicles following inhibition of TH synthesis among others (Opitz et al., 2012).

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Event: 281: Thyroxine (T4) in serum, Decreased

Short Name: T4 in serum, Decreased

Key Event Component

Process	Object	Action
abnormal circulating thyroxine level	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:194 - Hepatic nuclear receptor activation leading to altered amphibian metamorphosis	KeyEvent
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	KeyEvent

AOP ID and Name	Event Type
Aop:367 - Competitive binding to thyroid hormone carrier protein thyroid binding globulin (TBG) leading to altered amphibian metamorphosis	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	KeyEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	KeyEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:162 - Enhanced hepatic clearance of thyroid hormones leading to thyroid follicular cell adenomas and carcinomas in the rat and mouse	KeyEvent
Aop:128 - Kidney dysfunction by decreased thyroid hormone	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:192 - Pendrin inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:193 - Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole
Perchlorate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
serum

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-propylthouracil is a classic positive control for inhibition of TPO

Methimazole

Methimazole is a classic positive control for inhibition of TPO.

Perchlorate

Perchlorate ion (ClO_4^-) is a classic positive control for inhibition of NIS

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
chicken	Gallus gallus	Moderate	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI
Sus scrofa	Sus scrofa	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Taxonomic: This KE is plausibly applicable across vertebrates and the overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in zebrafish development, embryo-to-larval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002), and amphibian and lamprey metamorphoses is well established (Manzon and Youson, 1997; Yaoita and Brown, 1990; Furlow and Neff, 2006). Their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of THs in the different species depends on the expression and function of specific proteins (e.g receptors or enzymes) under TH control and may vary across species and tissues. As such, extrapolation regarding TH action across species and developmental stages should be done with caution.

With few exceptions, vertebrate species have circulating T4 (and T3) that are bound to transport proteins in blood. Clear species differences exist in serum transport proteins (Dohler et al., 1979; Yamauchi and Isihara, 2009). There are three major transport proteins in mammals; thyroid binding globulin (TBG), transthyretin (TTR), and albumin. In adult humans, the percent bound to these proteins is about 75, 15 and 10 percent, respectively (Schussler 2000). In contrast, in adult rats the majority of THs are bound to TTR. Thyroid- binding proteins are developmentally regulated in rats. TBG is expressed in rats until approximately postnatal day (PND) 60, with peak expression occurring during weaning (Savu et al., 1989). However, low levels of TBG persist into adult ages in rats and can be experimentally induced by hypothyroidism, malnutrition, or caloric restriction (Rouaze-Romet et al., 1992). While these species differences impact TH half-life (Capen, 1997) and possibly regulatory feedback mechanisms, there is little information on quantitative dose-response relationships of binding proteins and serum hormones during development across different species. Serum THs are still regarded as the most robust measurable key event causally linked to downstream adverse outcomes.

Life stage: The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, T4 levels are not expected to decrease in response to exposure to inhibitors of TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH system disruptors.

Sex: The KE is plausibly applicable to both sexes. THs are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of TH levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in TH levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Description

All iodothyronines are derived from the modification of tyrosine molecules (Taurog, 2000). There are two biologically active thyroid hormones (THs) in serum, triiodothyronine (T3) and T4, and a few less active iodothyronines (rT3, 3,5-T2). T4 is the predominant TH in circulation, comprising approximately 80% of the TH excreted from the thyroid gland in mammals and is the pool from which the majority of T3 in serum is generated (Zoeller et al., 2007). As such, serum T4 changes usually precede changes in other serum THs. Decreased thyroxine (T4) in serum results from one or more MIEs upstream and is considered a key biomarker of altered TH homeostasis (DeVito et al., 1999).

Serum T4 is used as a biomarker of TH status because the circulatory system serves as the major transport and delivery system for TH delivery to tissues. The majority of THs in the blood are bound to transport proteins (Bartalena and Robbins, 1993). In serum, it is the unbound, or 'free' form of the hormone that is thought to be available for transport into tissues. Free hormones are approximately 0.03 and 0.3 percent for T4 and T3, respectively. There are major species differences in the predominant binding proteins and their affinities for THs (see below). However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis across vertebrates (DeVito et al., 1999; Miller et al., 2009; Zoeller et al., 2007; Carr and Patiño, 2011).

Normal serum T4 reference ranges can be species and lifestage specific. In **rodents**, serum THs are low in the fetal circulation, increasing as the fetal thyroid gland becomes functional on gestational day 17, just a few days prior to birth. After birth serum hormones increase steadily, peaking at two weeks, and falling slightly to adult levels by postnatal day 21 (Walker et al., 1980; Harris et al., 1978; Goldey et al., 1995; Lau et al., 2003). Similarly, in **humans**, adult reference ranges for THs do not reflect the normal ranges for children at different developmental stages, with TH concentrations highest in infants, still increased in childhood, prior to a decline to adult levels coincident with pubertal development (Corcoran et al. 1977; Kapelari et al., 2008).

In some **frog** species, there is an analogous peak in THs in tadpoles that starts around embryonic NF stage 56, peaks at stage 62 and the declines to lower levels by stage 56 (Sternberg et al., 2011; Leloup and Buscaglia, 1977).

Additionally, ample evidence is available from studies investigating responses to inhibitors of TH synthesis in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

How it is Measured or Detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is the radioimmunoassay (RIA). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is commonly used as a human clinical test method. Analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, through methods employing HPLC, liquid chromatography, immuno luminescence, and mass spectrometry are less common, but are becoming increasingly available (Hornung et al., 2015; DeVito et al., 1999; Baret and Fert, 1989; Spencer, 2013; Samanidou V.F et al., 2000; Rathmann D. et al., 2015). In fish early life stages most evidence for the ontogeny of TH synthesis comes from measurements of whole body TH levels using LC-MS techniques (Hornung et al., 2015) are increasingly used to accurately quantify whole body TH levels as a proxy for serum TH levels (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020). It is important to note that TH concentrations can be influenced by a number of intrinsic and extrinsic factors (e.g., circadian rhythms, stress, food intake, housing, noise) (see for example, Döhler et al., 1979).

Any of these measurements should be evaluated for the relationship to the actual endpoint of interest, repeatability, reproducibility, and lower limits of quantification using a fit-for-purpose approach (i.e., different regulatory needs will require different levels of confidence in the AOP). This is of particular significance when assessing the very low levels of THs present in fetal serum. Detection limits of the assay must be compatible with the levels in the biological sample. All three of the methods summarized above would be fit-for-purpose, depending on the number of samples to be evaluated and the associated costs of each method. Both RIA and ELISA measure THs by an indirect methodology, whereas analytical determination is the most direct measurement available. All these methods, particularly RIA, are repeatable and reproducible.

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Event: 280: Thyroxine (T4) in neuronal tissue, Decreased

Short Name: T4 in neuronal tissue, Decreased

Key Event Component

Process	Object	Action
regulation of hormone levels	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent

Stressors

Name
Methimazole
Propylthiouracil

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

THs are critical for normal brain development in most vertebrates, primarily documented empirically in mammalian species (Bernal, 2013). However, there is compelling data that demonstrates the need for TH in brain development for many other taxa, including: birds, fish and frogs (Van Herck et al., 2013; Denver, 1998; Power et al., 2001). The most well known non-mammalian action of TH is to induce metamorphosis in amphibians and some fish species. However, there is a fundamental difference in the mechanisms by which T3 affects amphibian metamorphosis vs its role in mammalian brain development (Galton, 1983). In the rat, brain development proceeds, even if defective, despite the absence of TH. By contrast, TH administration to tadpoles induces early metamorphosis, whereas in its absence, tadpoles grow to extremely large size, but the metamorphosis program is never activated (Galton, 1983).

Key Event Description

Thyroid hormones (TH) are present in brain tissue of most vertebrate species, and thyroxine (T4) is converted to triiodothyronine (T3) locally in this tissue. The amount of THs in brain is known to vary during development and to differ among brain regions (Calvo et al., 1990; Kester et al., 2004; Tu et al., 1999). In human cerebral cortex, T3 increases steadily from 13-weeks, reaching adult levels by 20 weeks post conception. This occurs despite very low and unchanging levels in fetal serum T3, when fetal serum T4 increases 3-fold over the same period. This indicates that T3 in fetal brain is locally generated from serum-derived T4 via the activity of deiodinases, primarily DIO2. DIO2 serves to convert T4 to T3. During this time in fetal development DIO3 activity, which converts T3 to the inactive reverse T3 (rT3), remains very low in cortex. In contrast, in other brain regions including hippocampus and cerebellum, T3 remains low throughout early and mid-gestation and corresponds with high activity of DIO3 in these brain regions. In late gestation and after birth, DIO3 levels drop in hippocampus and cerebellum with a corresponding increase in T3 concentrations (Kester et al., 2004).

A similar spatial and temporal profile of deiodinase activity and corresponding brain hormone concentrations has been observed in rodent brain (Calvo et al., 1990; Tu et al., 1999). In the rat, either whole brain or cortex have been preferentially assessed due to the low levels of hormones present and the small tissue volumes make quantitification difficult. Brain T3 and T4 rise in parallel from gestational day 10 to gestational day 20 in rat. They are typically both quite low until gestational 17 with steep increases between GD18 and GD20 corresponding to the onset of fetal thyroid function (Calvo et al., 1990; Ruiz de Ono et al., 1988; Obergon et al., 1981). Just before birth, brain T3 and T4 concentrations are about one-third to one-half that of adult brain. Brain development in the early postnatal period in rat is roughly equivalent to the 3rd trimester in humans such that adult levels of T3 and T4 in brain are not reached in rodents until the 2nd-3rd postnatal week.

For THs to gain access to brain tissue they need to cross the blood brain barrier (BBB) which regulates the active transport of TH into neurons. Many transporter proteins have been identified, and the monocarboxylate transporters (Mct8, Mct10) and anion-transporting polypeptide (OATP1c1) show the highest degree of affinity towards TH and are prevalent in brain (Jansen et al., 2007; Mayer et al., 2014). Transporters express a distinct distribution pattern that varies by tissue and age (Friesema et al., 2005; Henneman et al., 2001; Visser et al., 2007; Heuer et al., 2005; Muller and Heuer, 2007). Although several transporters have been identified, current knowledge of cell specific profile of transporters is limited.

Most of the hormone transported across the blood brain barrier is in the form of T4, primarily through the cellular membrane transporters (e.g., OATP1c1 transporter) into the astrocyte (Visser and Visser, 2012; Sugiyama et al., 2003; Tohyama et al., 2004).

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 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.

How it is Measured or Detected

Radioimmunoassays (RIAs) are commonly used to detect TH in the brain (e.g., Obregon et al., 1982; Calvo et al., 1990; Morse et al., 1996; Bansal et al., 2005; Gilbert et al., 2013). The method (and minor variants) is well established in the published literature. However, it is not available in a simple 'kit' and requires technical knowledge of RIAs, thus has not been used in most routine toxicology studies. Evaluations in neuronal tissue are complicated by the difficulty of the fatty matrix, heterogeneity of regions within the brain, and low tissue concentrations and small tissue amounts especially in immature brain. Most often whole brain homogenates are assessed, obfuscating the known temporal and regional differences in brain hormone present. Two analytical techniques, LC- and HPLC-inductively coupled plasma–mass spectrometry have recently been used to measure brain concentrations of TH. These techniques have proven capable of measuring very low levels in whole-body homogenates of frog tadpoles at different developmental stages (e.g., Simon et al., 2002; Tietge et al., 2010). The assay detects I–, MIT, DIT, T4, T3, and rT3. More recently, Wang and Stapleton (2010) and Donzelli et al. (2016) used liquid chromatography-tandem mass spectrometry for the simultaneous analysis of five THs including thyroxine (T4), 3,3′,5-triidothyronine (T3), 3,3′,5′-triiodothyronine (rT3; reverse T3), 3,3′-diiodothyronine (3,3′-T2), and 3,5-diiodothyronine (3,5-T2) in serum and a variety of tissues including brain. These analytical methods require expensive equipment and technical expertise and as such are not routinely used.

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Event: 756: Hippocampal gene expression, Altered

Short Name: Hippocampal gene expression, Altered

Key Event Component

Process	Object	Action
regulation of gene expression	hippocampal formation	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:300 - Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent

Stressors

Name
Methimazole
Propylthiouracil

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rats	Rattus norvegicus	High	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High

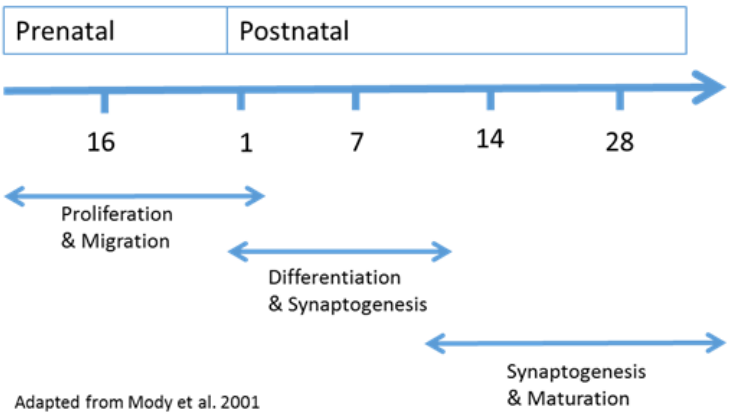
Sex	Evidence
Male	High

Gene expression in the developing brain in general is analogous across most mammalian species (Kempermann, 2012). Most of the empirical data on gene expression in hippocampus is from rat, mouse and human studies.

Key Event Description

Thyroid hormones control genes in the developing brain by classical ligand (T3) activation of thyroid receptors which leads to DNA binding and subsequent transcription and translation (for a review of TH rols in brain development see, Bernal 2015). Gene expression profiles have been published for the developing human and rodent hippocampus (Zhang et al., 2002; Mody et al., 2001). In both humans and rodents, the hippocampus undergoes typical stages of neurodevelopment found in most brain regions, including: cell proliferation, migration, differentiation, synapse formation, and the maturation of synaptic function. In the rodent, peak windows during pre- and post-natal periods have been identified during which major cellular and physiological events occur (see Figure 1). Each window expresses distinct patterns of gene transcription and clusters of genes increase their expression corresponding to the progression of events of hippocampal ontogeny (see Mody et al., 2001). Tables of gene clusters associated with these phases can be found in Supplementary Tables of Mody et al. (2001).

Figure 1. Mouse Hippocampal Developmental Stages Controlled by Gene Expression



During the very early prenatal period, genes corresponding to general cellular function are prominent (Mody et al., 2001). These are followed in time by genes regulating neuronal differentiation and migration in the mid to late gestational period. From late gestation (gestational day 15) until birth almost all the cells in the CA fields switch from a highly active proliferation state to a postmitotic state, and then undergo differentiation and migration. Expression of proliferative genes involved in cell cycle progression are highly expressed at gestational day 16, then subsequently are silent immediately after birth when genes directing neuronal growth switch on. The pyramidal neurons of the CA fields in the hippocampus proper develop in advance of the granule cells that comprise the principal cells of the dentate gyrus. As such, the genes controlling the distinct phases of neurodevelopment are expressed at different times in these two hippocampal subregions (Altman and Bayer, 1990a; b). In both subregions, however, many phenotypic changes within the hippocampal neuron occur in the period immediately after birth (postnatal day 1 to 7). Almost all neurons show extensive growth and differentiation during the first postnatal week. These cellular changes are marked by rapid cytoskeletal changes, production of cell adhesion molecules, and extracellular matrix formation. The gene families involved in these processes include actins, tubulins, and chaperonin proteins essential for promoting correct protein folding of cytoskeletal components. Cell adhesion and extracellular matrix proteins are also upregulated during this period as these genes are critical for differentiation and synaptogenesis.

During late postnatal hippocampal development (postnatal day 16-30), hippocampal circuits become more active and exhibit increased synaptic plasticity. Many genes upregulated during this phase of development are involved in synaptic function and include genes regulating vesicle associated proteins and calcium-mediated transmitter release, neurotrophins, and neurotransmitter receptors. Efficient energy utilization is essential during this period of increased synaptic activity, events mirrored by an upregulation of enzymes involved in glucose and oxidative metabolism.

How it is Measured or Detected

Measurement of genomic profiles in developing brain use methods that are well established and accepted in the published literature. Microarray studies with expression profile analyses have been conducted in cortex and hippocampus of humans (Zhang et al., 2002), non-human primates, and rodent brains of various ages (Mody et al., 2001; Royland et al., 2008; Dong et al., 2015). More commonly, quantitative rtPCR or in situ hybridization have been used to probe individual gene transcripts (Dowling et al.,

2000, Morte et al., 2010) or their protein products (Alvarez-Dolado et al., 1994; Gilbert et al., 2007). Recently RNA-Seq technology was applied to T3-treated primary mouse cortical cells and gene targets enriched in astrocytes and neurons to identify TH-responsive genes (Gil-Ibanez et al, 2015).

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Event: 757: Hippocampal anatomy, Altered

Short Name: Hippocampal anatomy, Altered

Key Event Component

Process	Object	Action
brain development	hippocampal formation	morphological change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent

AOP ID and Name	Event Type
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:300 - Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:442 - Inhibition of voltage gate sodium channels leading to impairment in learning and memory during development	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The hippocampus is generally similar in structure function across most mammalian species (West, 1990). The vast majority of information on the structure of the hippocampus is from mice, rats and primates including humans.

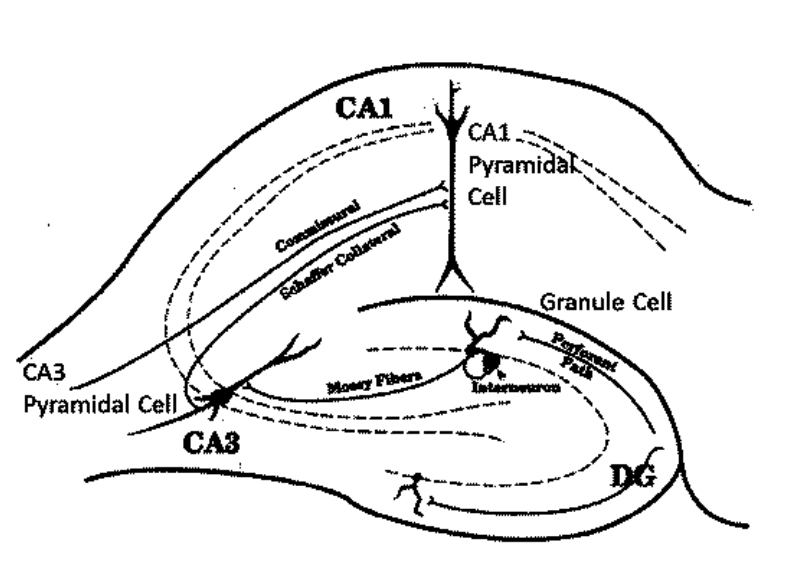
Key Event Description

The hippocampus is a major brain region located in the medial temporal lobe in humans and other mammals (West, 1990). Developmentally it is derived from neuronal and glial cells in the neural tube and differentiates in the proencephalon and telencephalon. The hippocampus is a cortical structure, but only contains 3-layers, distinct from the 6-layered neocortical structures. For this reason, it is known as archicortex or paleocortex meaning old cortex. Within humans, the structure is identified as early as fetal week 13 and matures rapidly until 2 to 3 years of age (Kier et al 1997), with continuing slow growth thereafter until adult ages (Utsunomiya et al., 1999). In rodents, the hippocampus begins to form in midgestation, with the CA fields forming in advance of the dentate gyrus. Dentate gyrus forms in late gestation with most of its development occurring in the first 2-3 postnatal weeks (Altman and Bayer, 1990a; 1990b).

The structure of the hippocampus has been divided into regions that include CA1 through CA4 and the dentate gyrus. The principal cell bodies of the CA field are pyramidal neurons, those of the dentate gyrus are granule cells. The dentate gyrus forms later in development than the CA fields of the hippocampus. These regions are generally found in all mammalian hippocampi.

The major input pathway to the hippocampus is from the layer 2 neurons of the entorhinal cortex to the dentate gyrus via the perforant path forming the first connection of the trisynaptic loop of the hippocampal circuit. Direct afferents from the dentate gyrus (mossy fibers) then synapse on CA3 pyramidal cells which in turn send their axons (Schaeffer Collaterals) to CA1 neurons to complete the trisynaptic circuit (Figure 1). From the CA fields information then passes through the subiculum entering the fiber pathways of the alveus, fimbria, and fornix and it routed to other areas of the brain (Amaral and Lavenex, 2006). 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. The primary input pathway to the CA regions of the hippocampus is from the septum by way of the fornix and direct input from the amygdala. Reciprocal outputs from the hippocampus back to these regions and beyond also exist.

Trisynaptic Hippocampal Circuitry



How it is Measured or Detected

Data in support of this key event have been collected using a wide variety of standard biochemical, histological and anatomical methods (e.g., morphometrics, immunohistochemical staining, in situ hybridization and imaging procedures). Many of methods applied to reveal anatomical abnormalities are routine neurohistopathology procedures similar to those recommended in EPA and OECD developmental neurotoxicity guidelines (US EPA, 1998; OCED, 2007). Subtle cytoarchitectural features depend on more specialized birth dating procedures and staining techniques. It is essential to consider the timing of events during development for detection to occur, as well as the timing for detection (Hevner, 2007; Garman et al., 2001; Zgraggen et al., 2012). Similar techniques used in rodent stydies have been applied to postmortem tissue in humans.

In humans, structural neuroimaging techniques are used to assess hippocampal volume with an analysis technique known as voxel-based morphometry (VBM). Volume of brain regions is measured by drawing regions of interest (ROIs) on images from brain scans obtained from magnetic resonance imaging (MRI) or positron emission tomography (PET) scans and calculating the volume enclosed. (Mechelli et al., 2005). Similar imaging techniques can be applied in rodent models (Powell et al., 2009; Hasegawa et al., 2010; Pirko et al., 2005; Pirko and Johnson, 2008).

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Event: 758: Hippocampal Physiology, Altered

Short Name: Hippocampal Physiology, Altered

Key Event Component

Process	Object	Action
chemical synaptic transmission	synapse	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:300 - Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent

Stressors

Name
Propylthiouracil
Iodine deficiency
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

The majority of evidence for this key event come from work in rodent species (i.e., rat, mouse). There is a moderate amount of evidence from other species, including humans (Clapp et al., 2012).

Key Event Description

The hippocampus functions as a highly integrated and organized communication and information processing network with millions of interconnections among its constitutive neurons. Neurons in the hippocampus and throughout the brain transmit and receive information largely through chemical transmission across the synaptic cleft, the space where the specialized ending of the presynaptic axon terminus of the transmitting neuron meets the specialized postsynaptic region of the neuron that is receiving that information (Kandell et al., 2012).

During development (see KE: Hippocampal anatomy, Altered), as neurons reach their final destination and extend axonal processes, early patterns of electrical synaptic activity emerge in the hippocampus. These are large fields of axonal innervation of broad synaptic target sites that are replaced by more elaborate but highly targeted and refined axonal projections brought about by activity-dependent synaptic pruning and synapse elimination. This is a classic case of the interaction between physiological and anatomical development, where anatomy develops first, and can be ‘reshaped’ by physiological function (Kutsarova et al., 2017).

In the rat, excitatory processes are fully mature in area CA1 of hippocampus within 2 weeks of birth with inhibitory processes lagging begin by several weeks (Muller et al., 1989; Michelson and Lothman, 1988; Harris and Teyler, 1984). In hippocampal slices, inhibitory function in areaCA1s is first seen on postnatal day 5 and increases in strength at postnatal day 12 through 15. In vivo studies fail to detect inhibition until postnatal day 18 with steady increase thereafter to adult levels by postnatal day 28. Synaptic plasticity in the form of long-term potentiation (LTP) is absent in the very young animal, only emerging about postnatal day 14, appearing to require the stability of both excitatory and inhibitory function to be established (Muller et al., 1989; Bekenstein and Lothman, 1991). These features of the maturation of hippocampal physiology are paralleled in dentate gyrus, but as with anatomical indices in the rat, the development of these physiological parameters lag behind the CA1 by about 1 week.

How it is Measured or Detected

In animals, synaptic function in the hippocampus has been examined with imaging techniques, but more routinely, electrical field potentials recorded in two subregions of the hippocampus, area CA1 and dentate gyrus, have been assessed in vivo or in vitro from slices taken from naive or exposed animals. Field potentials reflect the summed synaptic response of a population of neurons following direct stimulation of input pathways across a monosynaptic connection. Changes in response amplitude due to chemical perturbations and other stressors (e.g., iodine deficiency, thyroidectomy, gene knockouts) is evidence of altered synaptic function. This can be measured in vitro, in vivo, or in hippocampal slices taken from treated animals (Gilbert and Burdette, 1995). The most common physiological measurements used to assess function of the hippocampus are excitatory synaptic transmission, inhibitory synaptic transmission, and synaptic plasticity in the form of long-term potentiation (LTP).

Excitatory Synaptic Transmission: Two measures, the excitatory postsynaptic potential (EPSP) and the population spike are derived from the compound field potential at increasing stimulus strengths. The function described by the relationship of current strength (input, I) and evoked response (output, O), the I-O curve is the measure of excitatory synaptic transmission (Gilbert and Burdette, 1995).

Inhibitory Synaptic Transmission: Pairs of stimulus pulses delivered in close temporal proximity is used to probe the integrity of inhibitory synaptic transmission. The response evoked by the second pulse of the pair at brief intervals (<30 msec) arrives during the activation of feedback inhibitory loops in the hippocampus. An alteration in the degree of suppression to the 2nd pulse of the pair reflects altered inhibitory synaptic function (Gilbert and Burdette, 1995).

Long Term Potentiation (LTP): LTP is widely accepted to be a major component of the cellular processes that underlie learning and memory (Malenka and Bear, 2004; Bramham and Messaoudi, 2005). LTP represents, at the synapse and molecular level, the coincident firing of large numbers of neurons that are engaged during a learning event. The persistence of LTP emulates the duration of the memory. Synaptic plasticity in the form of LTP is assessed by delivering trains of high frequency stimulation to induce a prolonged augmentation of synaptic response. Probe stimuli at midrange stimulus strengths are delivered before and after application of LTP-inducing trains. The degree of increase in EPSP and PS amplitude to the probe stimulus after train application, and the duration of the induced synaptic enhancement are metrics of LTP. Additionally, contrasting I-O functions of excitatory synaptic transmission before and after (hours to days) LTP is induced is also a common measure of LTP maintanence (Bramham and Messaoudi, 2005; Kandell et al., 2012; Malenka and Bear, 2004).

Synaptic function in the human hippocampus has been assessed using electroencephalography (EEG) and functional neuroimaging techniques (Clapp et al., 2012). EEG is a measure of electrical activity over many brain regions but primarily from the cortex using small flat metal discs (electrodes) placed over the surface of the skull. It is a readily available test that provides evidence of how the brain functions over time. Functional magnetic resonance imaging or functional MRI (fMRI) uses MRI technology to measure brain activity by detecting associated changes in blood flow. This technique relies on the fact that cerebral blood flow and neuronal activation are coupled. Positron emission tomography (PET) is a functional imaging technique that detects pairs of gamma rays emitted indirectly by a radionuclide (tracer) injected into the body (Tietze, 2012; McCarthy, 1995). Like fMRI, PET scans indirectly measure blood flow to different parts of the brain – the higher the blood flow, the greater the activation (McCarthy, 1995). These techniques have been widely applied in clinical and research settings to assess learning and memory in humans and can provide information targeted to hippocampal functionality (McCarthy, 1995; Smith and Jonides, 1997; Willoughby et al., 2014; Wheeler et al., 2015; Gilbert et al., 1998).

Assays of this type are fit for purpose, have been well accepted in the literature, and are reproducible across laboratories. The assay directly measures the key event of altered neurophysiological function.

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List of Adverse Outcomes in this AOP

Event: 402: Cognitive Function, Decreased

Short Name: Cognitive Function, Decreased

Key Event Component

Process	Object	Action
learning or memory		decreased
cognition		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	AdverseOutcome
Aop:300 - Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:405 - Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	AdverseOutcome

Stressors

Name
Methimazole
Propylthiouracil
Iodine deficiency

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Basic forms of learning behavior such as habituation have been found in many taxa from worms to humans (Alexander, 1990). More complex cognitive processes such as executive function likely reside only in higher mammalian species such as non-human primates and humans.

Key Event Description

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. The main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990; Squire, 2004).

In humans, the hippocampus is involved in recollection of an event's rich spatial-temporal contexts and distinguished from simple semantic memory which is memory of a list of facts (Burgess et al., 2000). Hemispheric specialization has occurred in humans, with the left hippocampus specializing in verbal and narrative memories (i.e., context-dependent episodic or autobiographical memory) and the right hippocampus, more prominently engaged in visuo-spatial memory (i.e., memory for locations within an environment). The hippocampus is particularly critical for the formation of episodic memory, and autobiographical memory tasks have been developed to specifically probe these functions (Eichenbaun, 2000; Willoughby et al., 2014). In rodents, there is obviously no verbal component in hippocampal memory, but reliance on the hippocampus for spatial, temporal and contextual memory function has been well documented. Spatial memory deficits and fear-based context learning paradigms engage the hippocampus, amygdala, and prefrontal cortex (Eichenbaum, 2000; Shors et al., 2001; Samuels et al., 2011; Vorhees and Williams, 2014; D'Hooge and DeDeyn, 2001; Lynch, 2004; O'Keefe and Nadal, 1978). These tasks are impaired in animals with hippocampal dysfunction (O'Keefe and Nadal, 1978; Morris and Frey, 1987; Gilbert et al., 2016).

How it is Measured or Detected

In rodents, a variety of tests of learning and memory have been used to probe the integrity of hippocampal function. These include tests of spatial learning like the radial arm maze (RAM), the Barnes maze, and most commonly, the Morris water maze (MWM). Test of novelty such as novel object recognition, and fear based context learning are also sensitive to hippocampal disruption. Finally, trace fear conditioning which incorporates a temporal component upon traditional amygdala-based fear learning engages the hippocampus. A brief description of these tasks follows.

1) RAM, Barnes, MWM are examples of spatial tasks in which animals are required to learn: the location of a food reward (RAM); an escape hole to enter a preferred dark tunnel from a brightly lit open field area (Barnes maze); or a hidden platform submerged below the surface of the water in a large tank of water (MWM) (Vorhees and Williams, 2014).

2) Novel Object recognition. This is a simpler task that can be used to probe recognition memory. Two objects are presented to animal in an open field on trial 1, and these are explored. On trial 2, one object is replaced with a novel object and time spent interacting with the novel object is taken evidence of memory retention (i.e., I have seen one of these objects before, but not this one. Cohen and Stackman, 2015).

3) Contextual Fear conditioning is a hippocampal based learning task in which animals are placed in a novel environment and allowed to explore for several minutes before delivery of an aversive stimulus, typically a mild foot shock. Upon

reintroduction to this same environment in the future (typically 24-48 hours after original training), animals will limit their exploration, the context of this chamber being associated with an aversive event. The degree of suppression of activity after training is taken as evidence of retention, i.e., memory (Curzon et al., 2009).

4) Trace fear conditioning. Standard fear conditioning paradigms require animals to make an association between a neutral conditioning stimulus (CS, a light or a tone) and an aversive stimulus (US, a footshock). The unconditioned response (CR) that is elicited upon delivery of the footshock US is freezing behavior. With repetition of CS/US delivery, the previously neutral stimulus comes to elicit the freezing response. This type of learning is dependent on the amygdala, a brain region associated with, but distinct from the hippocampus. Introducing a brief delay between presentation of the neutral CS and the aversive US, a trace period, requires the engagement of the amygdala and the hippocampus (Shors et al., 2004).

Most methods are well established in the published literature and many have been engaged to evaluate the effects of developmental thyroid disruption. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426) both require testing of learning and memory (USEPA, 1998; OECD, 2007). These DNT Guidelines have been deemed valid to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009).

A variety of standardized learning and memory tests have been developed for human neuropsychological testing. These include episodic autobiographical memory, word pair recognition memory; object location recognition memory. Some components of these tests have been incorporated in general tests of adult intelligence (IQ) such as the WAIS and the Wechsler. Modifications have been made and norms developed for incorporating of tests of learning and memory in children. Examples of some of these tests include:

1) Rey Osterieth Complex Figure (RCFT) which probes a variety of functions including as visuospatial abilities, memory, attention, planning, and working memory (Shin et al., 2006).

2) Children's Auditory Verbal Learning Test (CAVLT) is a free recall of presented word lists that yields measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak 1995; Talley, 1986).

3) Continuous Visual Memory Test (CVMT) measures visual learning and memory. It is a free recall of presented pictures/objects rather than words but that yields similar measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak, 1984; 1994).

4) Story Recall from Wechsler Memory Scale (WMS) Logical Memory Test Battery, a standardized neurospychological test designed to measure memory functions (Lezak, 1994; Talley, 1986).

5) Autobiographical memory (AM) is the recollection of specific personal events in a multifaceted higher order cognitive process. It includes episodic memory- remembering of past events specific in time and place, in contrast to semantic autobiographical memory is the recollection of personal facts, traits, and general knowledge. Episodic AM is associated with greater activation of the hippocampus and a later and more gradual developmental trajectory. Absence of episodic memory in early life (infantile amnesia) is thought to reflect immature hippocampal function (Herold et al., 2015; Fivush, 2015).

6) Staged Autobiographical Memory Task. In this version of the AM test, children participate in a staged event involving a tour of the hospital, perform a series of tasks (counting footprints in the hall, identifying objects in wall display, buy lunch, watched a video). It is designed to contain unique event happenings, place, time, visual/sensory/perceptual details. Four to five months later, interviews are conducted using Children's Autobiographical Interview and scored according to standardized scheme (Willoughby et al., 2014).

Regulatory Significance of the AO

A prime example of impairments in cognitive function as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). In addition, testing for the impact of chemical expsoures on cognitive function, often including spatially-mediated behaviors, is an intergral part of both EPA and OECD developmental neurotoxicity guidelines (USEPA, 1998; OECD, 2007).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 309: Thyroperoxidase, Inhibition leads to TH synthesis, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Low
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	High	Low
Inhibition of thyroid peroxidase leading to impaired fertility in fish	adjacent	High	High
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	adjacent	High	Moderate
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	High	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: This KER is plausibly applicable across vertebrates. Inhibition of TPO activity is widely accepted to directly impact TH synthesis. This is true for both rats and humans, as well as some fishes, frogs and birds. Most of the data supporting a causative relationship between TPO inhibition and altered TH synthesis is derived from animal studies, *in vitro* thyroid microsomes from rats or pigs, and a limited number of human *ex vivo* (Nagasaka and Hidaka, 1976; Vickers et al., 2012) and clinical studies. There are data to support that gene mutations in TPO result in congenital hypothyroidism, underscoring the essential role of TPO in human TH synthesis.

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred THs during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnow, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH system disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to

males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroperoxidase (TPO) is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as the enzymatic catalyst for thyroid hormone (TH) synthesis (Taurog, 2005) across vertebrates. Two commonly used reference chemicals, propylthiouracil (PTU) and methimazole (MMI), are drugs that inhibit the ability of TPO to: a) activate iodine and transfer it to thyroglobulin (Tg) (Davidson et al., 1978); and, b) couple thyroglobulin (Tg)-bound iodotyrosyls to produce Tg-bound thyroxine (T4) and triiodothyronine (T3) (Taurog, 2005).

Evidence Supporting this KER

The weight of evidence supporting a direct linkage between the MIE, TPO inhibition, and the KE of decreased TH synthesis, is strong and supported by more than three decades of research in animals, including humans (Cooper et al., 1982; Cooper et al.,1983; Divi and Doerge, 1994).

Biological Plausibility

The biological plausibility for this KER is rated Strong. TPO is the only enzyme capable of de novo synthesis of TH. TPO catalyzes several reactions, including the oxidation of iodide, nonspecific iodination of tyrosyl residues of thyroglobulin (Tg) to form monoiodotyrosyl (MIT) or diiodotyrosyl (DIT) residues, and the coupling of these Tg-bound iodotyrosyls to produce Tg-bound T3 and T4 (Divi and Doerge, 1994; Kessler et al., 2008; Ruf et al., 2006; Taurog et al., 1996, 2005). Therefore, inhibition of TPO activity is widely accepted to directly impact TH synthesis.

Empirical Evidence

Empirical support for this KER is strong. There are several papers that have measured alterations in TPO and subsequent effects on TH synthesis across vertebrates. Taurog et al. (1996) showed decreased guicaol activity, decreased bound I^{125}, and subsequent decreases in newly formed T3 and T4 per molecule of Tg, following exposure to PTU, MMI and some antibiotics. There is important evidence in **mammals**. Following *in vivo* exposure to PTU in rats (Cooper et al., 1982; 1983), there are concentration and time-dependent decreases in thyroid protein bound iodine and serum T4 and T3 that recovered one month after cessation of PTU exposure. In addition, measures of thyroidal iodine content were highly correlated with intra-thyroidal PTU concentration. Vickers et al. (2012) demonstrated dose- and time- dependent inhibition of TPO activity in both human and rat thyroid homogenates exposed to MMI. Hassan et al. (2017, 2020) and Handa et al. (2021) predicted the level of THs in serum after treatment with PTU and MMI in rats. They developed a quantitative model by comparing dose- response data.

Tietge et al (2010) showed decreases in thyroidal T4 following MMI exposure in **Xenopus**. Also in Xenopus, Haselman et al (2020) showed decreases in thyroidal iodotyrosines (MIT/DIT) and iodothyronines (T4/T3) following exposure to MMI. Doerge et al (1998) showed that a tryphenylmethane dye, malachite green, inhibited TPO and lowered thyroxine production. A recent paper used a series of benzothiazoles and showed TPO inhibition (guicaol assay) and inhibition of TSH stimulated thyroxine release from Xenopus thyroid gland explant cultures (Hornung et al., 2015).

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). The impact of decreased TPO activity on TH synthesis is similar across all ages in mammals. Good evidence for the temporal relationship of the KER comes from thyroid system modeling (e.g., Degon et al., 2008; Fisher et al., 2013) using data from studies of iodine deficiency and chemicals that inhibit NIS. In addition, there is ample evidence of the temporal impacts of TPO inhibition on TH synthesis, using *ex vivo* and *in vitro* measures that demonstrate the time course of inhibition following chemical exposures, including some data from human thyroid microsomes and *ex vivo* thyroid slices (Vickers et al., 2012). Future work is needed that measures both TPO inhibition and TH production during development.

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal THs transferred to the eggs. Embryonic TH synthesis is activated later during embryo-

larval development. (See Domain of applicability)

Dose-Response Evidence: Dose-response data is available from a number of studies in **mammals** that correlate TPO inhibition with decreased TH production measured using a variety of endpoints including iodine organification (e.g., Taurog et al., 1996), inhibition of guicaol oxidation in thyroid microsomes (e.g., Doerge and Chang, 2002), and direct measure of thyroid gland T4 concentrations (e.g., Hornung et al., 2015). However, there is a lack of dose-response data from developmental studies showing direct linkages from TPO inhibition to thyroidal TH synthesis.

Uncertainties and Inconsistencies

While it is clear that TPO inhibition will lead to altered TH synthesis, there is a need for data that will inform quantitative modeling of the relationship between TPO inhibition and the magnitude of effects on TH synthesis.

Data from studies on genistein highlight this uncertainty. Doerge and colleagues have demonstrated that for this compound 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). Genistein is however a well-known phytoestrogen and the observed inconsistency may be the result of feedback mechanisms resulting from its estrogenic effect.

Quantitative Understanding of the Linkage

In *Xenopus laevis*, Haselman et al.(2020) demonstrated temporal profiles of thyroidal iodotyrosines (MIT/DIT) and iodothyronines (T4/T3), the products of TPO activity, following exposure to three different model TPO inhibitors (MMI, PTU, MBT) at multiple concentrations. This study established that, in *Xenopus*, measurable decreases in the products of TPO activity can occur as early as 2 days of exposure during pro-metamorphosis. However, despite consistent profiles of some iodo-species across chemicals, other iodo-species showed inconsistent profiles across chemicals. This highlights the multiple mechanisms of TPO (iodination and coupling) and differential susceptibility to inhibition of those mechanisms depending on the chemical's type of interaction with TPO. The most consistent concentration-response relationship across chemicals and over time was demonstrated by thyroidal T4, which is the most relevant product to subsequent key events. At the highest concentrations tested for each chemical, thyroidal T4 was below detection by 7 days of exposure across all three TPO inhibitors. Keeping in mind that the thyroid gland has follicular lumen space where thyroglobulin/T4 is stored until proteolysis and release to the blood, full inhibition of TPO would result in a delayed measurable response due to the time it takes to deplete stored hormones. Regardless of the delay, the results from this study imply full inhibition of TPO by each of these three chemicals at the highest test concentrations, but would require chemical residue analysis and/or toxicokinetic modeling to relate cellular/tissue concentrations at the site of TPO catalysis to levels of inhibition via Michaelis-Menten kinetic descriptions.

Profiles of thyroidal iodinated species demonstrated by Haselman et al. (2020) across three different TPO inhibitors suggests that a high level of TPO inhibition must occur in order to elicit responses in subsequent key events. Although the level of TPO inhibition is not directly quantifiable from this study, these data suggest that at least 90-100% inhibition was occurring since circulating T4 was not detectable at 10 days of exposure to the highest concentrations of MMI and MBT. However, additional efforts would be necessary to determine the minimum level of TPO inhibition that leads to a measurable decrease in thyroidal T4 and subsequently circulating T4. Furthermore, Hassan et al. (2017, 2020) and Handa et al. (2021) predicted the level of THs in serum after treatment with PTU and MMI in rats. They developed a quantitative model by comparing dose- response data.

Response-response relationship

There are only a limited number of studies where both TPO inhibition and iodine organification have been measured *in vivo*, and there is not enough data available to make any definitive quantitative correlations. One *in vivo* study in rats exposed to the TPO inhibitor genistein found no *in vivo* impact on serum TH concentrations, even when TPO was inhibited up to 80% (Chang and Doerge, 2000). Genistein is however a well-known phytoestrogen and the observed inconsistency may be the result of feedback mechanisms resulting from its estrogenic effect.

Given that this is an MIE to KE relationship, there is only one response to evaluate in the relationship. Decreased TH synthesis, as measured by responses of iodinated species in the thyroid gland, is the result of TPO inhibition, which cannot be measured directly *in vivo.*

Time-scale

In vivo, evaluations of TPO inhibition are limited to evaluation of the iodinated species, or products of TPO activity, present in the thyroid gland at a particular time. However, as stated previously, any measurable response in these iodinated species is not a discreet assessment of TPO activity given that the gland maintains storage of hormone in the follicular lumen space and any alteration of TPO activity would be detected once the stores begin to be depleted. In *Xenopus laevis*, Haselman et al. (2020) showed a decrease in thyroidal iodinated species after only 2 days of exposure to potent TPO inhibitor MMI during thyroid-mediated metamorphosis and within 4 days for PTU and MBT, both model TPO inhibitors. In zebrafish, Walter et al. (2019) reported a similar time frame, namely a decrease in T4 levels at 72 hpf after starting the exposure to PTU at 0-2 hpf. It should be noted that the time-scale is probably depending on the developmental stage and whether the embryo is capable of thyroid hormone synthesis, rather than on the exposure duration.

Known modulating factors

Iodine availability will impact the ability of TPO to iodinate tyrosine residues on thyroglobulin. Iodine availability to TPO can be

impacted in a number of ways. First, environmental availability of iodine can vary greatly depending on whether and how much iodine exists in surface waters for aquatic organisms (gill respirators) and in the diets of both terrestrial and aquatic organisms. Second, somewhat regardless of iodine availability through environmental uptake (i.e., barring extremely high iodine exposure), iodine is actively transported into the thyroid follicular cell from the blood via sodium-iodide symporter (NIS), which has been shown to be susceptible to inhibition by, for example, perchlorate. As such, iodine availability to TPO is mediated by functional NIS. Finally, iodine is not fully available to TPO on the apical surface of the thyroid follicular cell until it is transported through the apical membrane by pendrin, an anion exchange protein - mutations or inhibition of pendrin could affect iodine availability to TPO.

Hydrogen peroxide is also needed by TPO to mediate the oxidation of iodide, which is produced locally by dual oxidase (DUOX). A mutation or inhibition of DUOX will impact local production of H_2O_2 leading to lower oxidizing potential of TPO and less organification of iodide.

Known Feedforward/Feedback loops influencing this KER

Thyroid stimulating hormone (TSH) released from the pituitary positively regulates the synthesis and release of thyroid hormones from the thyroid gland. As such, when TPO is inhibited and thyroid hormone synthesis is decreased, lower systemic levels of hormone cause feedback from the pituitary via TSH to upregulate a number of processes in the thyroid gland as a means of compensation, including (but not limited to) enhanced gene expression of NIS and thyrocyte cell proliferation (Tietge et al., 2010; Haselman et al., 2020).

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Relationship: 305: TH synthesis, Decreased leads to T4 in serum, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	High
Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	adjacent	High	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		
Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		
Kidney dysfunction by decreased thyroid hormone	adjacent	High	
Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Pendrin inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
zebrafish	Danio rerio	Low	NCBI
fathead minnow	Pimephales promelas	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Male	High
Female	High

Taxonomic: This KER is plausibly applicable across vertebrates. While a majority of the empirical evidence comes from work with laboratory rodents, there is a large amount of supporting data from humans (with anti-hyperthyroidism drugs including propylthiouracil and methimazole), some amphibian species (e.g., frog), fish species (e.g., zebrafish and fathead minnow), and some avian species (e.g, chicken). The following are samples from a large literature that supports this concept: Cooper et al. (1982; 1983); Hornung et al. (2010); Van Herck et al. (2013); Paul et al. (2013); Nelson et al. (2016); Alexander et al. (2017); Stinckens et al. (2020).

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones (TH) during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH system disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) are synthesized by NIS and TPO in the thyroid gland as iodinated thyroglobulin (Tg) and stored in the colloid of thyroid follicles across vertebrates. Secretion from the follicle into serum is a multi-step process. The first involves thyroid stimulating hormone (TSH) stimulation of the separation of the peptide linkage between Tg and TH. The next steps involve endocytosis of colloid, fusion of the endosome with the basolateral membrane of the thyrocyte, and finally release of TH into blood. More detailed descriptions of this process can be found in reviews by Braverman and Utiger (2012) and Zoeller et al. (2007).

Evidence Supporting this KER

The weight of evidence linking these two KEs of decreased TH synthesis and decreased T4 in serum is strong. It is commonly accepted dogma that decreased synthesis in the thyroid gland will result in decreased circulating TH (serum T4).

Biological Plausibility

The biological relationship between two KEs in this KER is well understood and documented fact within the scientific community.

Empirical Evidence

It is widely accepted that TPO inhibition leads to declines in serum T4 levels in adult **mammals**. This is due to the fact that the sole source for circulating T4 derives from hormone synthesis in the thyroid gland. Indeed, it has been known for decades that insufficient dietary iodine will lead to decreased serum TH concentrations due to inadequate synthesis. Strong qualitative and quantitative relationships exist between reduced TH synthesis and reduced serum T4 (Ekerot et al., 2013; Degon et al., 2008; Cooper et al., 1982; 1983; Leonard et al., 2016; Zoeller and Tan, 2007). There is more limited evidence supporting the relationship between decreased TH synthesis and lowered circulating hormone levels during development. Lu and Anderson (1994) followed the time course of TH synthesis, measured as thyroxine secretion rate, in non-treated pregnant rats and correlated it with serum T4 levels. Modeling of TH in the rat fetus demonstrates the quantitative relationship between TH synthesis and serum T4 concentrations (Hassan et al., 2017, 2020; Handa et al., 2021). Furthermore, a wide variety of drugs and chemicals that inhibit TPO are known to result in decreased release of TH from the thyroid gland, as well as decreased circulating TH concentrations. This is evidenced by a very large number of studies that employed a wide variety of techniques, including thyroid gland explant cultures, tracing organification of 131-I and *in vivo* treatment of a variety of animal species with known TPO inhibitors (King and May,1984; Atterwill et al., 1990; Brown et al., 1986; Brucker-Davis, 1998; Haselman et al., 2020; Hornung et al., 2010; Hurley et al., 1998; Kohrle, 2008; Tietge et al., 2010).

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on

T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). There are currently no studies that measured both TPO synthesis and TH production during development. However, the impact of decreased TH synthesis on serum hormones is similar across all ages in mammals. Good evidence for the temporal relationship comes from thyroid system modeling of the impacts of iodine deficiency and NIS inhibition (e.g., Degon et al., 2008; Fisher et al., 2013). In addition, recovery experiments have demonstrated that serum thyroid hormones recovered in athyroid mice following grafting of in-vitro derived follicles (Antonica et al., 2012). In *Xenopus*, it has been shown that depression of TH synthesis in the thyroid gland precedes depression of circulating TH within 7 days of exposure during pro-metamorphosis (Haselman et al., 2020).

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal THs transferred to the eggs. Embryonic TH synthesis is activated later during embryo-larval development. (See Domain of applicability)

Dose-response Evidence: Dose-response data is lacking from studies that include concurrent measures of both TH synthesis and serum TH concentrations. However, data is available demonstrating correlations between thyroidal TH and serum TH concentrations during gestation and lactation during development (Gilbert et al., 2013). This data was used to develop a rat quantitative biologically-based dose-response model for iodine deficiency (Fisher et al., 2013). In *Xenopus*, dose-responses were demonstrated in both thyroidal T4 and circulating T4 following exposure to three TPO inhibitors (Haselman et al., 2020).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. The first uncertainty stems from the paucity of data for quantitative modeling of the relationship between the degree of synthesis decrease and resulting changes in circulating T4 concentrations. In addition, most of the data supporting this KER comes from inhibition of TPO, and there are a number of other processes (e.g., endocytosis, lysosomal fusion, basolateral fusion and release) that are not as well studied.

For example, Kim et al. (2015) investigated the adverse effects of Triphenyl phosphate (TPP), a substance that disrupts the thyroid system. Therefore, **Rat pituitary** (GH3) and **thyroid follicular cell lines** (FRTL-5) were studied. In the GH3 cells, TPP led to an upregulation of the expression of important thyroid genes (tsh, tr alpha and tr beta) while T3, a positive control, downregulated the expression of these genes. In FRTL-5 cells, the expression of nis and tpo genes was significantly upregulated, suggesting that TPP stimulates TH synthesis in the thyroid gland.

In **zebrafish larvae** at the age of 7 days post-fertilisation (dpf), TPP exposure resulted in a significant **increase in T3 and T4** concentrations and the expression of genes involved in thyroid hormone synthesis. Exposure to TPP also significantly regulated the expression of genes involved in the metabolism (dio1), transport (ttr) and excretion (ugt1ab) of THs. The down-regulation of the crh and tsh genes in the zebrafish larvae suggests the activation of a central regulatory feedback mechanism that is triggered by the increased T3 levels in vivo. Taken together, these observations indicate that TPP increases TH concentrations in early life stages of zebrafish by disrupting central regulatory and hormone synthesis pathways.

Quantitative Understanding of the Linkage

In rats, Hassan et al. (2020) demonstrated *in vitro: ex vivo* correlations of TPO inhibition using PTU and MMI and constructed a quantitative model relating level of TPO inhibition with changes in circulating T4 levels. They determined that 30% inhibition of TPO was sufficient to decrease circulating T4 levels by 20%. This is further supported by studies of Hassan et al. (2017) and Handa et al. (2021)

In *Xenopus*, Haselman et al. (2020) collected temporal and dose-response data for both thyroidal and circulating T4 which showed strong qualitative concordance of the response-response relationship. A quantitative relationship exists there in, but is yet to be demonstrated mathematically in this species.

Response-response relationship

Fisher et al. (2013) published a quantitative biologically-based dose-response model for iodine deficiency in the rat. This model provides quantitative relationships for thyroidal T4 synthesis (iodine organification) and predictions of serum T4 concentrations in developing rats. There are other computational models that include thyroid hormone synthesis. Ekerot et al. (2012) modeled TPO, T3, T4 and TSH in dogs and humans based on exposure to myeloperoxidase inhibitors that also inhibit TPO. This model was recently adapted for rats(Leonard et al., 2016) and Hassan et al (2017) have extended it to include the pregnant rat dam in response to TPO inhibition induced by PTU. While the original model predicted serum TH and TSH levels as a function of oral dose, it was not used to explicitly predict the relationship between serum hormones and TPO inhibition, or TH synthesis. Leonard et al.

(2016) recently incorporated TPO inhibition into the model. Degon et al (2008) developed a human thyroid model that includes TPO, but does not make quantitative prediction of organification changes due to inhibition of the TPO enzyme. Further empirical support for the response-response relationship has been demonstrated in the amphibian model, *Xenopus laevis*, exposed to TPO inhibitors during pro-metamorphosis (Haselman et al., 2020) wherein temporal profiles were measured for both thyroidal and circulating T4.

Time-scale

Given that the thyroid gland contains follicular lumen space filled with stored thyroglobulin/T4, complete inhibition of thyroid hormone synthesis at a given point in time will not result in an instantaneous decrease in circulating T4. The system will be capable of maintaining sufficient circulating T4 levels until the gland stores are depleted. The time it takes to deplete stored hormone will greatly depend on species, developmental status and numerous other factors.

In *Xenopus*, Haselman et al. (2020) demonstrated an approximately 5 day difference between a significant decrease in thyroidal T4 preceding a significant decrease in circulating T4 while exposed to a potent TPO inhibitor (MMI) continuously during pro-metamorphosis.

Known modulating factors

During *Xenopus* metamorphosis, circulating T4 steadily increases to peak levels at metamorphic climax. Therefore, during *Xenopus* metamorphosis, this KER is operable at an increased rate as compared to a system that is maintaining steady circulating T4 levels through homeostatic control. In this case, developmental status is a modulating factor for the rates and trajectories of these KEs.

Known Feedforward/Feedback loops influencing this KER

This KER is entirely influenced by the feedback loop between circulating T4 originating from the thyroid gland and circulating TSH originating from the pituitary. Intermediate biochemical processes exist within the hypothalamus to affirm feedback and coordinately release TSH from the pituitary. However, quantitative representations of these feedback processes are limited to models discussed previously.

In *Xenopus*, circulating levels of T4 increase through pro-metamorphosis indicating a "release" of feedback to allow circulating levels of T4 to increase and drive metamorphic changes (Sternberg et al., 2011). This provides evidence that homeostatic control of feedback can be developmentally dependent, and likely species dependent.

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Relationship: 312: T4 in serum, Decreased leads to T4 in neuronal tissue, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Low
Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	adjacent	Moderate	Low
Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High
All life stages	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

The majority of the information on this KER comes from in vivo studies with rodents (mainly MCT8 knock-out mice and thyroidectomized rats) and histopathological analyses of human brain tissues derived from patients affected by AHDS (Allan-Herndon-Dudley syndrome). The evoluationary conservation of the transport of TH from circulation to the developing brain suggests, with some uncertainty, that this KER is also applicable to other mammalian species.

Key Event Relationship Description

In mammals, thyroxine (T4) in brain tissue is derived almost entirely from the circulating pool of T4 in blood. Transfer of free T4 (and to a lesser extent, T3) from serum binding proteins (thyroid binding globulin (TBG), transthyretin (TTR) and albumin; see McLean et al., 2017, for a recent review) into the brain requires transport across the blood brain barrier (BBB) and /or indirect transport from the cerebral spinal fluid (CSF) into the brain through the blood-CSF-barrier. The blood vessels in rodents and humans expresses the main T4 transporter, MCT8, (Roberts et al. 2008), as does the choroid plexus which also expresses TTR and secretes the protein into the CSF (Alshehri et al. 2015).

T4 entering the brain through the BBB is taken up into astrocytes via cell membrane iodothyronine transporters (e.g., organic anion-transporting polypeptides OATP), monocarboxylate transporter 8 (MCT8) (Visser et al., 2011). 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 (MCT8) 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 eventually 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).

Evidence Supporting this KER

The weight of evidence linking reductions in circulating serum TH and reduced brain concentrations of TH is moderate. Many studies support this basic linkage. However, there are compensatory mechanisms (e.g., upregulation of deiodinases, transporters) that may alter the relationship between hormones in the periphery and hormone concentrations in the brain. There is limited information available on the quantitative relationship between circulating levels of TH, these compensatory processes, and neuronal T4 concentrations, especially during development. Furthermore, in certain conditions, such as iodine deficiency, the decreases in circulating hormone might have greater impacts on tissue levels of TH (see for instance, Escobar del Rey, et al., 1989).

Biological Plausibility

The biological relationship between these two KEs is strong as it is well accepted dogma within the scientific community. There is no doubt that decreased circulating T4 leads to declines in tissue concentrations of T4 and T3 in a variety of tissues, including brain. However, compensatory mechanisms (e.g., increased expression of Type 2 deiodinase) may differ during different lifestages and across different tissues, especially in different brain regions. Similarly, the degree to which serum TH must drop to overwhelm these compensatory responses has not been established.

Empirical Evidence

Several studies have shown that tissue levels, including brain, of TH are proportional to serum hormone level (Oppenheimer, 1983; Morreale de Escobar et al., 1987; 1990; Calvo et al., 1992; Porterfield and Hendrich, 1992, 1993; Broedel et al., 2003). In thyroidectomized rats, brain concentrations of T4 were decreased and Type II deiodinase (DII) activity was increased. Both brain T3 and T4 as well as DII activity returned to normal following infusion of T4 (Escobar-Morreale et al., 1995; 1997). Animals treated with PTU, MMI, or iodine deficiency during development demonstrate both lower serum and lower brain TH concentrations (Escobar-Morreale et al 1995; 1997; Taylor et al., 2008; Bastian et al., 2012; 2014; Gilbert et al., 2013). Compared to the wildtype, a mouse MCT8 knockout model has was shown to have decreased plasma T4, decreased uptake of T4 into the brain, and decreased brian T3 concentrations, as well as increased cortical diodinase Type 2 activity and increased plasma T3 concentrations (Mayerl et al., 2014; Barez-Lopez et al., 2016).

> *Temporal Evidence:* The temporal relationship between serum T4 and T4 in growing neuronal tissue described in this KER is dependent on the developmental stage (Seed et al., 2005). While all brain regions will be impacted by changes in serum hormones, brain concentrations will be a function of development stage and brain region. Data are available from thyroid hormone replacement studies that demonstrate recovery of fetal brain T3 and T4 levels (following low iodine diets or MMI

exposure) to control levels after maternal thyroid hormone replacement or iodine supplementation (e.g., Calvo et al.,1990; Obregon et al., 1991). For example, Calvo et al. (1990) carried out a detailed study of the effects of TPO inhibition on serum and tissues levels of TH in gestating rats. Clear dose-dependent effects of T4 replacement, but not T3 replacement were seen in all maternal tissues. However, for fetal tissues, neither T4 nor T3, at any dose, could completely restore tissue TH levels to control levels.

Dose-Response Evidence: There is good evidence, albeit from a limited number of studies of the correlative relationship between circulating thyroid hormone concentrations and brain tissue concentrations during fetal and early postnatal development following maternal iodine deficient diets or chemical treatments that depress serum THs (c.f., Calvo et al., 1990; Obregon et al., 1991; Morse et al.,1996).

Uncertainties and Inconsistencies

The fact that decreased serum TH results in lower brain TH concentrations is well accepted. However, the ability of the developing brain to compensate for insuffiencies in serum TH has not been well studied. Limited data is available that demonstrates that changes in local deiodination in the developing brain can compensate for chemical-induced alterations in TH concentrations (e.g., Calvo et al., 1990; Morse et al., 1996; Sharlin et al., 2010). And, there are likely different quantitative relationships between these two KEs depending on the compensatory ability based on both developmental stage and specific brain region (Sharlin et al., 2010). For these reasons, the empirical support for this linkage is rated as moderate

The role of cellular transporters represents an additional uncertainly. In addition, future work on cellular transport mechanisms and deiodinase activity is likley to inform addition of new KEs and KERs between serum and brain T4.

Quantitative Understanding of the Linkage

Response-response relationship

While it is well established that decreased in serum TH levels result in decreased brain TH concentrations, particularly fetal brain concentrations, a major gap is the lack of empirical data that allow direct quantification of this relationship (Hassan et al., 2018). Recently, serum TH and brain TH were measured in fetal cortex and postnatal day 14 offspring following graded degrees of hypothyroidism induced by PTU (O'Shaughnessy et al., 2018). Results showed that brain levels TH levels at both ages were quantitatively related to serum T4 levels. Additional dose-response information is necessary to confirm these findings, and standardization of analysis for the measurements in these distinct matrices is crucial to allow comparisons to be made between independent experiments.

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Relationship: 746: T4 in neuronal tissue, Decreased leads to Hippocampal gene expression, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Most of the data available has come from rodent models. The evolutionary conservation of thyroid receptors (Holzer et al., 2017) coupled with their role in TR regulated gene transcription in neurodevelopment, suggests that this KER may also be applicable to other species (see text above).

Key Event Relationship Description

Many cellular and biochemical effects of thyroid hormones (TH) are mediated through regulation of gene expression (Oppenheimer, 1983; Bernal, 2007). Thyroxine (T4) is transferred from the serum to the brain (see KER: Thyroxine (T4) in Serum, Decreased leads to Thyroxine (T4) in Neuronal Tissue, Decreased), where it converted to triiodothyronine (T3), the level of which is highly controlled by deiodinases. T3 binds to thyroid receptors (TR) in the nucleus of neuronal and glial cells to control gene expression. It is generally accepted that the modulation of TR gene expression in the hippocampus, or any other brain region, must therefore depend on the presence of hormone in these tissues.

Evidence Supporting this KER

The weight of evidence is moderate for TH concentrations affecting gene expression in the developing brain is (Oppenheimer and Schwartz, 1997; Oppenheimer, 1983; Bernal, 2007; Morte et al., 2010a; 2010b; Williams, 2008). Direct measurement of TH in brain tissue, and in hippocampus in particular, has shown correlations with gene expression. Therefore, it is assumed that reductions in TH-responsive genes in the hippocampus stem from reduced availability of hormone in the brain from the serum. However, studies in which there are simultaneous assessments of hippocampal concentrations of thyroid hormone and hippocampal gene expression is limited.

Biological Plausibility

The biological relationship between these two KEs is strong. It is a generally accepted fact that TH produce their actions on brain development by binding to nuclear receptors to affect gene transcription. See KER (1387): "T4 in serum, Decreased leads *(non-adjacently)* to Hippocampal gene expression, Altered" for more information on TR regulated genes. As the primary means whereby TH promotes its action is by binding to TR in brain, TH must be present in brain to affect this action. Circulating levels of T4 represent the primary source of T4 in the brain, which is then converted to the active hormone T3 by deiodinases within neuronal tissue.

Empirical Evidence

The empirical support for this KER is moderate. Many in vitro studies have demonstrated a relationship between hormone concentraionsTH and the induction of gene expression in brain cells, including hippocampal neurons in culture (Gil-Ibanez et al., 2015; Morte et al., 2010b). However, there are a limited number of studies investigating TH concentrations in the hippocampus and hippocampal gene expression. This is the case because thyroid hormone is difficult to measure in hippocampus and TH-induced gene expression changes can be subtle. We are aware of only four in vivo studies in which both thyroid hormones in the brain and gene expression in brain were simultaneously measured (Bastian et al., 2012; 2014; Hernandez et al., 2010; Sharlin et al., 2008). Only two of these reports, stemming from the same laboratory, specifically assessed thyroid hormone and gene expression in hippocampus. In these studies, Bastian et al., (2012; 2014) measured decrements in hippocampal T3 using RIA and correlated these reductions with alterations in the expression of myelin associated genes (Mbp, Plp), the neurotrophin, Ngf, the calcium binding protein Parv, a TH-dependent transcription factor, Hr, and Agt.

> *Temporal Evidence:* The temporal nature of this KER on TH dependent gene regulation is developmental (Seed et al., 2005). The impact of brain TH concentrations on regulation of TR regulated genes is age-dependent for a number of genes critical for normal hippocampal development. It is widely accepted that different genes are altered dependent upon the window of exposure in the fetal, neonatal or adult brain (c.f., Pathak et al, 2011; Mohan et al., 2012; Quignodon et al., 2004; Williams, 2008). Thyroid hormone supplementation has been shown to reverse some of the effects on gene expression (Mohan et al., 2012; Liu et al., 2010; Pathak et al., 2011).
>
> *Dose-Response Evidence:* Dose-response data exists but is limited to a small number of studies and a small number of genes (Bastian et al., 2012; 2014; Sharlin et al., 2008).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are uncertainties. Uncertainties remain in the relationship of neuronal TH concentrations and gene expression in the brain because of the lack of studies simultaneously examining brain hormone and gene expression in the same study. This stems from the technological challenges associated with measuring brain hormone and the sometimes-subtle changes in brain gene expression induced by manipulations of the thyroid system. In addition, there are also some physiological actions of T4 that are mediated non-genomically at the cell membrane (Davis et al., 2016). However, the exact role for the non-genomic effects is not well accepted or understood (Galton, 2017).

Quantitative Understanding of the Linkage

Response-response relationship

There is only one study available to date that provides empirical data on both TH concentrations and measures of gene expression changes in brain. O'Shaughnessy et al (2018) demostrates dose-response relationships between brain T4 and T3 concentrations and changes in a variety of genes (e.g., Parv, Col11a2, Hr, Ngf) that were "statistically significant at doses that decreased brain t4 and/or T3". There was no quantitation of this relationship reported.

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Relationship: 747: Hippocampal gene expression, Altered leads to Hippocampal anatomy, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Moderate	NCBI

Life Stage Applicability			
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability	
Life Stage	**Evidence**
During brain development	Moderate

Sex Applicability

Sex	Evidence
Male	High
Female	High

The majority of data in support of this KER is from rodent models. The evolutionary conservation of thyroid receptors (Holzer et al., 2017) coupled with their role in TR regulated gene transcription in neurodevelopment, suggests that this KER may also be applicable to other species.

Key Event Relationship Description

The basic biological processes that link gene regulation in the structural formation and function of all organs of the body are similar throughout the developing organism. In the developing brain, genes encode proteins critical for developmental events intrinsic to structural development (e.g., neurogenesis, neuronal migration, synaptogenesis, myelination). The development of the hippocampus is no exception to this general rule of biology.

Evidence Supporting this KER

The overall weight of evidence is moderate for a direct linkage between perturbation of the expression of genes in brain (and in hippocampus specifically) and neuroanatomical abnormalities. It is widely acknowledged that the development of the structure of the hippocampus is under the control of hippocampal gene expression. However, while an extensive body of literature exists linking some genes to hippocampal structure, there is no complete compendium on the total number of genes involved, nor direct causative links between the myriad of genes and the intricate development (both timing and location) of the majority of hippocampal structure.

Biological Plausibility

The biological plausibility of this KER is rated as strong. It is well established that gene regulation controls brain development. This also applies to the development of the hippocampus, where nuclear thyroid receptors that regulate gene transcription, directly or indirectly via transcription factor regulation, to control translation.

Empirical Evidence

Empirical support for this KER is rated as strong. The number of publications in this area is extensive. A few examples are: Strange et al. (2014); Takei et al. (2016); and Shin et al. (2015). Work supporting the relationship includes use of a variety of animal models (i.e., nutritional deficiencies, chromosome abnormalities, gene deletions, knock out animals, toxicant exposures and developmental hormonal imbalance) (e.g., Frotscher, 2010; Castren and Castren, 2014; Spilker et al., 2016; Skucas et al., 2011; Lessman et al., 2011). Mutant mouse lines generated for genes involved in human cortical malformations such as doublecortin, reelin, Lis1 and Tuba1a also show gross disorganization within the hippocampus (Khalaf-Nazzal et al., 2013). Collectively, data from these studies clearly support the link between alterations in hippocampal gene expression and structural changes in hippocampal volume, cell number, and/or cytoarchitecture. A direct linkage between some specific gene targets and structural change in the hippocampus has been demonstrated using knock out and mutant mouse models (e.g., Grant et al., 1992; Lee et al., 2000; Frotscher, 2010; Castren and Castren, 2014; Spilker et al., 2016; Skucas et al., 2011; Lessman et al., 2011; Khalaf-Nazzal et al., 2013).

> *Temporal Evidence*: The temporal nature of this KER is developmental (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of TR-regulated genes and subsequent formation of the anatomy of the hippocampus. This has been demonstrated in multiple studies. Many of the gene-anatomy relationships critical to brain development only exist during development, or exist only to a very limited extent in the adult brain. For example, genes controlling neuronal proliferation and migration are critically essential in hippocampal development, and their disruption results in abnormal hippocampal anatomy. Whereas, in the adult brain the genes are largely without effect as these processes are completed in the early neonatal period. In support of this, a limited number of studies have defined critical periods for the interaction of some genes and resulting neuroanatomical organization of the hippocampus (Lee et al., 2015; Favaro et al., 2009; Lee et al., 2000). In addition, there are some 'rescue' experiments for a select number of genes (eg., Lee et al., 2015; Spilker et al., 2016). Several examples are described below:
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> In the Jacob/Nsfm knockout model, hippocampal dysplasia is seen in hippocampal areas CA1 and CA3, characterized by reduced complexity of the synapto-dendritic cytoarchitecture, shorter dendrites and fewer branches (Spilker et al., 2016). Simplified dendritic trees and reduced synaptogenesis were also observed in hippocampal primary neurons cultured from these knock out mice relative to cultures from wild type mice. The protein product of Jacob/Nsfm regulates activity-dependent brain-derived neurotrophic factor (*Bdnf*) transcription. Lower BDNF levels were seen in area CA1 of knock out mice on postnatal day 10. The dysplasia seen in hippocampal neuronal cultures from knock mice could be reversed by BDNF supplementation if administered in early (2-4 days in vitro) but not later (15 days in vitro) in development.

Neuroregluin-2 (*Nrg2*) contributes to synaptogenesis of the granule cell layer of the hippocampus. In hippocampal slice cultures, inducible microRNA targeting strategies have demonstrated early suppression of *Nrg2* (4 days in vitro) but not late suppression (7 days in vitro) reduced synaptogenesis of inhibitory neurons. On the other hand, late treatment impaired the dendritic outgrowth of excitatory synaptic connections. These effects could be eliminated with overexpression of *Nrg2* (Lee et al., 2015).

Many of the gene-regulated processes involved in hippocampal development are also present in the developing cortex. In models of prenatal hypothyroidism, altered expression patterns of many genes involved in neuronal migration and apoptosis are associated with disruptions in hippocampal organization and cytoarchitecture of the cerebral cortex (Pathak et al, 2011; Mohan et al., 2012; Lui et al., 2010). Structural changes in hippocampus and cerebral cortex are dependent on time of exposure (Auso et al., 2003; Berbel et al., 2010; Pathak et al., 2011) and can be reversed with TH supplementation (Mohan et al., 2012; Pathak et al., 2011; Berbel et al., 2010).

Dose-Response Evidence: Dose-response data is lacking for this KER. Papers that utilize knock-out and mutant models do not provide 'dose-response' information for gene-anatomy relationships. Studies in which genes and anatomy were reported following developmental hypothyroidism were single high-dose studies that focused on varying the developmental window of exposure, but not necessarily the dose.

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. Few studies exist that report both gene expression changes and structural changes in the hippocampus in same study to provide direct causative evidence for this KER. Lacking also is the specific suite of genes that are altered in the hippocampus at particular developmental times that are causal to the structural defects reported. For future research, it is critical to generate data in which the upstream KE is modulated in a 'dose-response' manner to better support the causative relationship. Significant data gaps also exist for basic fetal hippocampal development.

Quantitative Understanding of the Linkage

Response-response relationship

There are no data on the quantitative linkages between gene expression changes and altered hippocampal anatomy.

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Pathak A, Sinha RA, Mohan V, Mitra K, Godbole MM. 2011. Maternal thyroid hormone before the onset of fetal thyroid function regulates reelin and downstream signaling cascade affecting neocortical neuronal migration. Cerebral Cortex.11-21.

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Relationship: 749: Hippocampal anatomy, Altered leads to Hippocampal Physiology, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low
Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
human	Homo sapiens	Not Specified	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The majority of data in support of this KER is from rodent models. The evolutionary conservation of hippocampal anatomy in mammals, birds, and reptiles (see Hevner, 2016; Streidter, 2015) suggests, with some uncertainty, that this KER is also applicable to multiple species.

Key Event Relationship Description

The hippocampus is a highly integrated and organized communication and information processing network with millions of interconnections among its constitutive neurons (see Andersen et al, 2006). The neuronal spine is the primary site of action for synaptic interface between neurons. Although difficult to measure due to their small size, large number and variable shapes, changes in the frequency and structure of dendritic spines of hippocampal neurons has dramatic effects on synaptic physiology and plasticity (Harris et al., 1992). Anatomical integrity at a more macro-level is also essential for physiological function. The connectivity of axons emanating from one set of cells that synapse on the dendrites of the receiving cells must be intact for effective communication between neurons to be possible. Synaptogenesis is a critical step for neurons to be integrated into neural networks during development. Changes in the placement of cells within the network due to delays or alterations in neuronal migration, the absence of a full proliferation of dendritic arbors and spine upon which synaptic contacts are made, and the lagging of transmission of electrical impulses due to insufficient myelination will independently and cumulatively impair synaptic function.

Evidence Supporting this KER

The weight of evidence supporting the relationship between structural abnormalities in brain induced and altered synaptic function is moderate. There is no doubt that altered structure can lead to altered function. Many examples from knock out models, genetic mutations, prenatal alcohol, nutritional deficits demonstrate a correlative link between altered structure and impaired synaptic function within the hippocampus (Gil-Mohapel et al., 2010; Berman and Hannigan, 2000; Grant et al., 1992; Palop et al., 2010; Ieraci and Herrera, 2007). However, the scientific understanding of the causative and quantitative relationship between the two KEs is incomplete.

Biological Plausibility

The biological plausibility of alterations in hippocampal structure having an impact on synaptic function and plasticity in brain is strong. Because synaptic transmission in the hippocampus relies on the integrity of contacts and the reliability of electrical and chemical transmission between pre- and post-synaptic neurons, it is well accepted that interference on the anatomical levels will very much impact the functional output on the neurophysiological level (Knowles, 1992; Schultz and Engelhardt, 2014).

Empirical Evidence

Empirical support for this KER is rated as moderate. Numerous examples of a direct linkage between hippocampal anatomy and hippocampal physiology are evident in knock out or transgenic mouse models (eg., Lessman et al., 2011). Other data is derived from nutritional deficiencies, alcohol exposure, and hippocampal slice culture models (Berman and Hannigan, 2000; Ieraci and Herrera, 2007; Gilbert et al., 2016). Although several examples are evident to demonstrate direct linkages between alterations in hippocampal anatomy and disruptions in hippocampal physiology, there is not a mechanism, anatomical insult, or signature pattern of synaptic impairment that accompanies each of these treatments.

Below are a few examples where direct linkages have been reported and they serve to bear witness to a direct relationship between altered hippocampal anatomy and altered hippocampal physiology.

Fyn is a tyrosine kinase gene involved in synaptic plasticity. Mutations of this gene lead to a lack of expression during development and result in an increase in the number of neurons in the dentate gyrus and CA subfields of the hippocampus. Fyn mutant mice also exhibited impairments in long term potentiation in hippocampal CA1 whereas two other forms of short-term plasticity remained intact (Grant et al., 1992).

Neuroregluin-2 (NRG2) is a growth factor and is highly expressed in the hippocampal dentate where it contributes to synaptogenesis of newborn granule cells. In hippocampal slice cultures, inducible microRNA targeting strategies have demonstrated suppression of NRG2 reduced synaptogenesis of inhibitory neurons and impaired dendritic outgrowth and maturation of glutamatergic synapses. These anatomical alterations were accompanied by reductions in the amplitude of excitatory synaptic currents. The magnitude of the impairment was dependent on the timing of the infection and could be eliminated with overexpression of NRG2 in this in vitro model (Lee et al., 2015).

Brain-derived neurotrophic factor (BDNF) activation of CREB-activated gene expression plays a documented role in hippocampal synapogenesis, dendrite formation, and synaptic plasticity in the developing and adult nervous system (Lessmann et al., 2011; Panja and Bramham, 2014). Jacob is a protein that translocates to the nucleus upon activation of BDNF-dependent pathways and is involved in both neuronal plasticity and neurodegeneration. Hippocampal neurons in culture derived from Jacob/Nsmf knockout mice exhibit shorter neurite length, reduced branching, and a few synaptic contacts. This effect was specific to hippocampal neurons, as cortical cells derived from the same animals did not display these abnormalities. In vivo, these animals exhibited a reduction of dendritic complexity of CA1 neurons, lower number of branches, decreased spine density. Deficits in synaptic plasticity in the form of LTP accompanied these structural impairments (Spilker et al., 2016).

In Alzheimer's Disease, amyloid-b protein accumulates in the hippocampus and leads to the formation of amyloid plaques, neuritic dystrophy and aberrant sprouting of axon terminals of the hippocampus. In a developmental germ-line knockout mouse model, high levels of amyloid-b induced aberrant neuronal network excitability and altered innervation of inhibitory interneurons. Deficits in

hippocampal plasticity were seen in the dentate gyrus without change in basal levels of synaptic transmission. In contrast, in area CA1, synaptic transmission was impaired while measures of synaptic plasticity remained intact (Palop et al., 2007).

Other evidence for a direct linkage between hippocampal anatomy and hippocampal physiology comes from the area of adult neurogenesis. The neurogenesis process refers to the acquisition of new neurons on the hippocampus of the adult brain and is associated with enhanced hippocampal synaptic function and learning ability (Deng et al., 2010). Manipulations such as caloric restriction, exercise and hormones can enhance neurogenesis and increase synaptic transmission and plasticity (Kapoor et al., 2015; Trivino-Paredes et al., 2016; Deng et al., 2010). A reciprocal relationship also exists whereby increases in hippocampal neural activity serves to increase neurogenesis (Bruel-Jungerman et al., 2007, Bruel-Jungerman et al., 2009, Kameda et al., 2012). Manipulations that decrease hippocampal neurogenesis including exposure to antidepressants, hormone disruption, stress, and alcohol are associated with impaired synaptic function (Herrera et al., 2003; Saxe et al., 2006; Gilbert et al., 2016; Montero-Pedrazuela et al., 2006; Gil-Mohapel et al., 2006; Sofroniew et al., 2006).

> *Temporal Evidence:* The temporal nature of this KER is developmental (Seed et al., 2005). This has been demonstrated in multiple studies. A few examples detailed above defined critical periods for the manipulation that alters the structural development of the hippocampus that persists to adulthood to disrupt the synaptic physiology measured in the hippocampus in adulthood (Lee et al., 2015; Grant et al., 1992). A more limited number of ‘rescue’ experiments have been reported. Lee et al (2015), using an in vitro model, demonstrated impaired synaptogenesis that was dependent on the timing of the infection and could be eliminated with overexpression of NRG2. In Spliker et al (2016), BDNF application rescued the morphological deficits in hippocampal pyramidal neurons from Jacob/Nsmf mice.
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> *Dose-Response Evidence:* Dose-response data is lacking for this KER. For future research, it is critical to generate data in which the upstream KE is modulated in a ‘dose-response’ manner to better support the causative relationship.

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are uncertainties. Although several examples are evident to demonstrate direct linkages between alterations in hippocampal anatomy and disruptions in hippocampal physiology, there is not a common cellular mechanism, anatomical insult, or signature pattern of synaptic impairment that defines a common anatomically driven physiological phenotype. In addition, it is also known that there is an interaction between physiological and anatomical development, where anatomy develops first, and can be ‘reshaped’ by the ongoing maturation of physiological function (e.g., Kutsarova et al., 2017)

Quantitative Understanding of the Linkage

Response-response relationship

Information does not exist to develop quantitative relationships between the KEs in this KER. Papers that utilize knock-out and mutant models have not provided ‘dose-response’ information for anatomy-physiology relationships.

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Relationship: 748: Hippocampal Physiology, Altered leads to Cognitive Function, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
humans	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The majority of data in support of this KER is from rodent models. The evolutionary conservation of the role of the hippocampus in spatial cognitive functions suggests, with some uncertainty, that this KER is also applicable to other mammalian species.

Key Event Relationship Description

It is a well-accepted assertion that hippocampal synaptic integrity and plasticity are essential for spatial information processing in animals and spatial and episodic memory in humans (Burgess, 2002; Martin et al., 2000; Sweatt, 2016). A large number of studies with a variety of techniques and approaches have linked hippocampal functional deficits to decreased spatial ability, context learning, and fear learning. Study of human disease states and conditions where hippocampal function is impaired (i.e., brain trauma, Alzheimer's disease, temporal lobe epilepsy, Down's Syndrome), and imaging studies of hippocampal activation during memory challenge, makes itirrefutable that the hippocampus is essential for specific types of cognition abilities. Decades of animal research has reinforced this assertion.

There are many forms of synaptic plasticity and numerous ways in which physiological function of neural circuits can be assessed. Similarly, there are many forms of learning and memory and multiple tasks and specifics associated with these tasks that vary from laboratory to laboratory. An emerging field of computational cognitive neuroscience lies at the intersection of computational neuroscience, machine learning and neural network theory. These computational and theoretical frameworks support the participation of the hippocampal synaptic transmission and plasticity in learning and memory in animals and humans (for review see: Ashby and Helie, 2012).

Evidence Supporting this KER

The weight of evidence for proper hippocampal function and episodic memory in humans and the animal analogue, spatial and fear-based context learning, is strong. Seminal studies over the past 60 years firmly established the cellular basis of behavior with synaptic plasticity (LTP and LTD). And recent work has provided details on the local hippocampal circuitry needed for memory formation and behavioral change (Sweatt, 2016). In humans, virtual reality experiments in large-scale spatial contexts demonstrate the convergence of spatial memory performance in normal patients with fMRI of the hippocampus clearly demonstrating the essentiality of hippocampal function to spatial learning (Burgess, 2002). This assertion is consistent with a wealth of animal data on hippocampal learning and memory. In rodent models, functional impairment of the hippocampus assessed using electrophysiological techniques is correlated with deficits in spatial memory typically assessed using mazes, and memory for context often assessed in fear-based learning paradigms (O'Keefe and Nadel, 1978; Clark et al., 2000; Squire, 2004; Eichenbaum, 2000; Panjo and Bramham, 2014).

Biological Plausibility

The biological plausibility of the KER is rated as strong. It is well accepted that the normal hippocampal function is critical for the acquisition and memory of context and spatially mediated tasks in rodents and humans (Sweatt, 2016).

Empirical Evidence

Empirical support for this KER is strong. The requisite of hippocampal integrity to optimal visuo-spatial context learning (i.e., episodic memory) in humans and spatial learning in rodents is well documented. In vivo recording in conscious behaving animals

has demonstrated activity-dependent neural changes taking place in the hippocampus during spatial learning (Gruart and Delgado-Garcia, 2007). Impairments in hippocampal function induced by drugs, chemicals, lesions, mutant or knock out models that cause changes in synaptic transmission, plasticity, and hippocampal network activity, are coincident with deficits in spatial and context-based fear learning (O'Keefe and Nadel, 1978; Bannerman et al., 2014; Lynch, 2004; Verret et al., 2012). Similarly, treatments found to enhance or facilitate hippocampal synaptic transmission and plasticity are associated with improved learning and memory (Deng et al., 2010; Novkovic et al., 2015; Andrade et al., 2015; Trivino-Paredes et al., 2016). For example, n-methyl-d-aspartate (NMDA)-mediated glutamatergic synaptic transmission is essential for the induction of hippocampal synaptic plasticity in the form of LTP. Blockade of this form of plasticity by selective NMDA-receptors blockers impairs LTP and hippocampal tests of learning and memory (reviewed in Sweatt, 2016). Perturbation of hippocampal plasticity and impaired spatial learning have been reported in adult offspring following prenatal ethanol exposure (An and Zhang, 2015). The fyn mutant mouse (fyn is a tyrosine kinase pathway) displays impairments in hippocampal synaptic transmission and plasticity, as well asspatial learning deficits (Grant et al., 1992). Brain-derived neurotrophic factor (BDNF) knock out animals exhibit synaptic plasticity deficits and learning impairments (Aarse et al., 2016; Panja and Bramham, 2014). In the Jacob/Nfsm model which also exhibits pronounced alterations in BDNF-mediated signaling, hippocampal synaptic transmission and plasticity impairments were accompanied by deficits in contextual fear conditioning and novel location recognition tasks (Spilker et al., 2016). Finally, in rodent models of developmental TH insufficiency, impairments in hippocampal synaptic transmission and plasticity are coincident with deficits in learning tasks that require the hippocampus (Opazo et al., 2008; Gilbert and Sui, 2006, Gilbert, 2011, Gilbert et al., 2016).

In humans, hippocampal physiology assessed using neuroimaging reveals activation of hippocampus upon engagement in spatial learning and episodic memory providing a direct linkage of these two specific KEs (Burgess, 2002). In fMRI studies of congenitally hypothyroid children, or children born to women with altered thyroid function during pregnancy, changes in hippocampal activity patterns during memory encoding and retention were observed and associated with memory impairments (Wheeler et al., 2012; 2015; Willoughby et al., 2013; 2014).

> *Temporal Evidence:* The temporal nature of this KER is developmental (Seed et al., 2005). This has been demonstrated in multiple studies. It is well-recognized that there are critical developmental windows for disruption of the functional development of the hippocampus and the integrity of this structure is essential for later development of spatial ability, context learning, and fear learning. A wealth of studies have shown correlation between hippocampal LTP and spatial learning performance, as well as the role of glutamatergic synaptic transmission and BDNF-mediated signaling pathways in these processes (Bramham, 2007; Andero et al., 2014; Morris et al., 1986; Sweatt, 2016; Migaud et al., 1998). Although studies on reversibility are rare, deficits in hippocampal synaptic transmission and plasticity in slices from BDNF knockout animals can be rescued with recombinant BDNF (Patterson et al., 1996).
>
> *Dose-Response Evidence:* Limited dose-response information is available. Studies have investigated dose-dependency of both electrophysiological and behavioral impairments in animals suffering from developmental TH insufficiency (e.g., Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2016).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. It is a widely-held assertion that synaptic transmission and plasticity in the hippocampus underlie spatial learning (Martin et al., 2000; Gruart and Delgado-Garcia, 2007; Bramham, 2007). 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.

Quantitative Understanding of the Linkage

Response-response relationship

Information does not exist to develop quantitative relationships between the KEs in this KER.

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List of Non Adjacent Key Event Relationships

Relationship: 366: Thyroperoxidase, Inhibition leads to T4 in serum, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	non-adjacent	High	Low
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	non-adjacent	High	High
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Xenopus laevis	Xenopus laevis	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	Moderate	NCBI
human	Homo sapiens	High	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: Use of TPO inhibitors as anti-hyperthyroidism drugs in humans and pets (Emiliano et al., 2010; Trepanier, 2006) and effects of these drugs on serum TH concentrations in rats (US EPA, 2005), amphibian, fish and avian species (Coady et al., 2010; Grommen et al., 2011; Nelson et al., 2016; Rosebrough et al., 2006; Stinckens et al.; 2020; Tietge et al., 2012), strongly supports a causative linkage between inhibition of TPO and decreased serum T4 across species. Therefore, this KER is plausibly applicable across vertebrate species. Therefore, this KER is plausibly applicable across vertebrates.

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the

formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body TH levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). Therefore, iIt is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH system disruptors.

Sex: The KE is plausibly applicable to both sexes. THs are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of TH levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in TH levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroperoxidase (TPO) is the enzyme that catalyzes iodine organification of thyroglobulin to produce thyroglobulin (Tg)-bound T3 and T4 in the lumen of thyroid follicles. Tg-bound THs are endocytosed across the apical lumen-follicular cell membrane, undergo thyroglobulin proteolysis, followed by hormone section into the blood stream (see Taurog, 2005 for review). This indirect KER describes the relationship of TPO inhibition to reduced circulating levels of thyroid hormone (TH) in the serum.

Evidence Supporting this KER

The weight of evidence linking thyroperoxidase inhibition to reductions in circulating serum TH is strong. Many studies support this basic linkage. There is no inconsistent data.

Biological Plausibility

It is a well-accepted fact that inhibition of the only enzyme capable of synthesizing THs, TPO, results in subsequent decrease in serum TH concentrations. A large amount of evidence from clinical and animal studies clearly support the commonly accepted dogma that inhibition of TPO leads to decreased serum THs.

Empirical Evidence

The majority of research in support of this KER involves exposure to known TPO inhibitors and measurement of serum hormones. There are many in vivo studies that link decreases in serum TH concentrations with exposure to xenobiotics that inhibit thyroperoxidase (TPO) in **mammals** (Brucker-Davis, 1998; Hurley, 1998; Boas et al., 2006; Crofton, 2008; Kohrle, 2008; Pearce and Braverman, 2009; Murk et al., 2013).

While these studies support the connection between exposure to a known TPO inhibitor and decreased TH, many of these studies do not empirically measure TPO inhibition or decreased TH synthesis. Thus, many studies support the indirect linkage between TPO inhibition (for chemicals identified as TPO inhibitors in in vivo or ex vivo studies) and decreased TH, with the well accepted theory that these proceed via decreased TH synthesis. That exposure to TPO inhibitors leads to decreased serum TH concentrations, via decreased TH synthesis is strongly supported by decades of mechanistic research in a variety of species.

This indirect relationship is also evidenced by the use of clinically-relevant anti-hyperthyroidism drugs, MMI and PTU (Laurberg & Anderson, 2014; Sundaresh et al., 2013). These drugs are both recognized TPO inhibitors and are part of a standard drug-based regimen of care for clinically hyperthyroid patients including those with Grave's disease. Serum THs are measured as the bioindicator of successful treatment with anti-hyperthyroidism drugs; the actual decrease in TH synthesis in the thyroid gland is implied in the efficacious use of these drugs (Trepanier, 2006).

In **rats**, MMI and PTU are often used as control chemicals to decrease serum THs to study biological phenomena related to disruption of TH homeostasis (many examples, including Zoeller and Crofton, 2005; Morreale de Escobar et al, 2004; Schwartz et al., 1997; Herwig et al., 2014; Wu et al., 2013; Pathak et al., 2011). Further, MMI is recommended as a positive control for use in the **Amphibian** Metamorphosis (Frog) Assay within Tier 1 of the U.S. EPA Endocrine Disruptor Screening Program (US EPA, 2009; Coady et al., 2010), an assay used to evaluate the potential for chemicals to disrupt TH homeostasis. PTU has been suggested as positive control chemical in the guidance for the Comparative Developmental Thyroid Assay (US EPA, 2005), a non-guideline assay used to evaluate the potential for chemicals to disrupt TH homeostasis during gestation and early neonatal development.

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al.,

2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Thus, an indirect key event relationship between TPO inhibition and decreased serum THs is strongly supported by a large database of clinical medicine and investigative research with whole animals (with a great deal of supporting evidence in rats and frogs).

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). The qualitative impact of TPO inhibition on serum hormones is similar across all ages in mammals. The temporal nature of the impact on serum THs by TPO inhibitors in developmental exposure studies is evidenced by the duration of exposure and developmental age (Goldey et al., 1995; Ahmed et al., 2010; Tietge et al., 2010), as well as recovery after cessation of exposure (Cooke et al., 1993; Goldey et al., 1995; Sawin et al., 1998; Axelstad et al., 2008; Shibutani et al., 2009; Lasley and Gilbert, 2011). The temporal relationship between TPO inhibitor exposure duration and serum hormone decreases in adult organisms has been widely demonstrated (e.g., Hood et al., 1999; Mannisto et al., 1979). In addition, MMI and PTU induced decreases in serum T4 are alleviated by TH replacement in both fetal and postnatal age rats (Calvo et al., 1990; Sack et al., 1995; Goldey and Crofton, 1998). Computational modeling of the thyroid also provides evidence for the indirect temporal relationship between these two KEs (e.g., Degon et al., 2008; Fisher et a., 2013).

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Dose-Response Evidence: Empirical data is available from enough studies in animals treated with TPO inhibitors during development to make it readily accepted dogma that a dose-response relationship exists between TPO inhibition and serum TH concentrations. Again, these studies do not empirically measure TPO inhibition or decreased TH synthesis, but rely on the strong support of decades of mechanistic research in a variety of species of the causative relationship between these KEs. Examples of dose-responsive changes in TH concentrations following developmental exposure to TPO inhibitors include studies a variety of species, including: rodents (Blake and Henning, 1985; Goldey et al., 1995; Sawin et al., 1998); frogs (Tietge et al., 2013); fish tissue levels (Elsalini and Rohr, 2003.); and, chickens (Wishe et al., 1979). Computational modeling of the thyroid also provides evidence for the indirect dose-response relationship between these two KEs (e.g., Leonard et al., 2016; Fisher et a., 2013).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. The predominant uncertainty regarding the indirect key event relationship between inhibition of TPO activity and decreased serum T4 is the quantitative nature of this relationship, i.e., to what degree must TPO be inhibited in order to decrease serum T4 by a certain magnitude. Many animal (rat) studies typically employ relatively high exposures of TPO-inhibiting chemicals that result in hypothyroidism (severe decrements in T4 and T3). Thus, a dose-response relationship between TPO inhibition and decreased serum T4 is not typically defined. However, there are numerous publications demonstrating clear dose- and duration- dependent relationships between TPO inhibitors dose and reduced serum T3 and T4 in rodent models (see for example: Cooper et al., 1983; Hood et al., 1999; Goldey et al., 2005; Gilbert, 2011). The relationship between maternal and fetal levels of hormone following chemically-induced TPO inhibiton has not been well characterized and may differ based on kinetics. Reductions in serum TH in the fetus, in rats and humans is derived from a chemical's effect on the maternal thyroid gland as well as the fetal thyroid gland.

Quantitative Understanding of the Linkage

Hassan et al. (2017, 2020) and Handa et al. (2021) worked out a quantitative model to predict serum T4 levels based on TPO inhibition in rats.

Response-response relationship

The indirect linkage between exposure to known TPO inhibitors and decreased serum TH has not been defined quantitatively. The two key event relationships that mediate this relationship (TPO inhibition leading to decreased TH synthesis, and decreased TH synthesis leading to decreased serum TH) have been incorporated into some quantitative models. A quantitative biologically-based dose-response model for iodine deficiency in the rat includes relationships between thyroidal T4 synthesis and serum T4 concentrations in developing rats Fisher et al. (2013). Ekerot et al. (2012) modeled TPO, T3, T4 and TSH in dogs and humans based on exposure to myeloperoxidase inhibitors that also inhibit TPO and was recently adapted for rat (Leonard et al., 2016). While the original model predicted serum TH and TSH levels as a function of oral dose, it was not used to explicitly predict the relationship between serum hormones and TPO inhibition, or TH synthesis. Leonard et al. (2016) recently incorporated TPO inhibition into the model. Degon et al (2008) developed a human thyroid model that includes TPO but does not make quantitative prediction of organification changes due to inhibition of the TPO enzyme.

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Relationship: 1387: T4 in serum, Decreased leads to Hippocampal gene expression, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Most of the data available has come from rodent models.

Key Event Relationship Description

Many of the physiological effects of thyroid hormones (THs) are mediated through regulation of gene expression by zinc finger nuclear receptor proteins that are encoded by thyroid hormone genes alpha (Thra) and beta (Thrb). It is widely accepted that TH regulates gene transcription during brain development (Bernal, 2007; Anderson et al., 2003). The sole source of TH to the brain is from the circulating levels of the prohormone, thyroxine (T4). Once taken up from the serum to reach the brain, T4 is converted to triiodothyronine (T3) which binds to TH nuclear receptors (TRα and TRβ). On binding, and in the presence of regulatory cofactors, transcription of certain genes is either up- or down-regulated (Oppenheimer, 1983). However, only a small number of genes have been shown to be directly influenced by TH receptor binding, and of these, most are transcription factors (Quignodon et al., 2008; Thompson and Potter, 2000; Horn and Heuer, 2010). In this manner, THs do influence a wide variety of genes.

Evidence Supporting this KER

The weight of evidence for this indirect relationship is strong. It is well established that serum TH is the primary source of brain T4 from which neuronal T3, the active hormone, is locally generated and presented to the receptors in the nucleus of neurons to control gene transcription.

Biological Plausibility

The biological plausibility of this KER is rated as strong. This is consistent with the known biology of the relationship between serum TH concentrations and brain TH concentrations, and the known action of TH to mediate gene transcription in brain and many other tissues.

Empirical Evidence

The empirical support for this KER is strong. A global transcriptome analysis of primary cerebrocoritical cells was recently published in which a number of genes regulated by T3 were identified (Gil-Ibanez et al., 2015). Although the bulk of literature in which serum TH reductions have been associated with gene expression changes in the brain have been focused on the cortex, several reports in hippocampus are available. Genes directly regulated by TH include the transcription factors Hr and Klf9 (Bteb) (Thompson and Potter, 2000; Cayrou et al., 2002; Denver and Williamson, 2009). The expression of a number of genes modulated by TH are expressed in the hippocampus. Many of genes that regulate processes involved in hippocampal development are also present in the developing cortex. Thus, Table 1 lists TH responsive genes whose expression in either area are altered by TH reduction. This list is not meant to be exhaustive, just exemplary.

Gene Name	Tissue	Model	Age	Reference
FETAL				
Klf9 (Bteb)	Rat- Cortex	MMI+CLO4	Fetus-GD17	Dong et al., 2015
Nurr1	Mouse-cortex	Thyroidectomy, MMI + ClO4	Fetal GD17; PN90	Navarro et al., 2014
Bdnf	Rat- Cortex	MMI	Fetus GD14-18	Pathak et al., 2011
Trkb	Rat- Cortex	MMI	Fetus GD14-18	Pathak et al., 2011
MCT8	Rat- Cortex	MMI	Fetus GD14-18	Mohan et al, 2012
Dio2	Rat -Cortex	MMI	Fetus GD14-18	Mohan et al, 2012
CyclinD1	Rat- Cortex	MMI	Fetus GD14-18	Mohan et al, 2012
Cyclin D2	Rat- Cortex	MMI	Fetus GD14-	Mohan et al, 2012

Cyclin D2	Rat- Cortex	MMI	18	Mohan et al, 2012
Pax6	Rat- Cortex	MMI	Fetus GD14	Mohan et al, 2012
Hr	Mouse- cortex	MMI + ClO4	Fetal GD17	Morte et al., 2010
Sema7a	Mouse- Cortex	MMI + ClO4	Fetal GD17	Morte et al., 2010
RC3 (Neurogranin)	Rat- Hippocampus, Cortex	MMI	Fetus-GD16	Dowling and Zoeller, 2001
Camk4	Mouse-cortex	Thyroidectomy, MMI + ClO4	Fetal GD17; PN90	Morte et al., 2010; Navarro et al., 2014
NEONATAL				
Klf9 (Bteb)	Rat, Mouse- Cortex	PTU	Neonate-PN14	Royland et al., 2008; Bastian et al., 2012; Denver and Williamson, 2009; Denver et al., 1999
Hr	Rat- Cortex, Hippocampus, Cerebellum	PTU, MMI	Neonate-PN14	Royland et al., 2008; Bastian et al., 2012; Thompson and Potter, 2000; Morte et al., 2010
Parv	Rat- cortex	PTU	Neonate-PN14/21	Royland et al., 2008; Bastian et al., 2012; 2014, Shiraki et al., 2014
Ngf	Rat- Hippocampus, cortex	PTU	Neonate-PN14, PN90	Royland et al., 2008; Bastian et al., 2012; Gilbert et al., 2016
Agt	Rat- Cortex	PTU	Neonate, PN14	Royland et al., 2008; Bastian et al., 2012; 2014
Col11a2	Rat- Cortex	PTU	Neonate, PN14	Royland et al., 2008
Itih2	Rat- Cortex	PTU	Neonate, PN14	Royland et al., 2008
Sema7a	Rat- Cortex	PTU	Neonate-PN14	Royland et al., 2008
Reelin	Rat- Hippocampus, cortex, cerebellum	Thyroidectomy, PTU		Alvarez-Dolado et al. 1999; Shiraki et al., 2014
Mbp	Rat- Hippocampus, Cortex, Cerebellum	PTU, MMI	Neonate-PN14/21	Ibarrola et al., 1997; Royland et al., 2008; Bastian et al., 2012; 2014, Shiraki et al, 2014
Plp2	Hippocampus, Cortex	PTU, MMI		Royland et al., 2008; Bastian et al., 2012
Camk4	Rat/Mouse- cortex	PTU	Neonate-PN14	Royland et al., 2008
RC3 (Neurogranin)	Rat-hippocampus	Thyroidectomy + MMI	Neonate-PN5, PN21	Iniquez et al., 1993; Dong et al., 2010

Temporal Evidence: The temporal nature of this KER is developmental (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered gene expression in the developing brain, including the hippocampus. Rescue experiments for this endpoint of gene expression in hypothyroid models are limited. In one, a combination of T3 and T4 treatment delivered on the last day of a 3-day gestational MMI hypothyroxinemia mouse model altered the pattern of gene expression observed in the cortex of offspring relative to euthyroid controls and MMI alone (Dong et al., 2015).

Dose-Response Evidence: There are a limited number of studies that have reported on the dose-dependent nature of the correlation between serum THs and hippocampal gene expression (Bastian et al., 2012; 2014; Royland et al., 2008).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. It is widely accepted that changes in serum THs will result in alterations in hippocampal gene expression. Several different animal models have been used to manipulate serum TH

concentrations that also measure gene expression changes. Varying windows of exposure to TH disruption and developmental sample time and region examined have also varied across studies. However, dose-response data is lacking. Most investigations of hippocampal gene expression have employed treatments that induce severe hormone reductions induced by PTU or MMI, or by thyroidectomy. In addition, few reports have studied the genes in the hippocampus, the cortex being more accessible in young animals. Finally, when the hippocampus is the target, different genes at different ages are reported, making it difficult to compare findings.

Quantitative Understanding of the Linkage

Response-response relationship

There are no quantitative models that predict the degree of serum TH reduction that is required to alter hippocampal gene transcription. Most investigations for hippocampus have been conducted in the neonate after severe hormone reductions. Only four publications have reported dose-dependent effects on gene expression in at less than maximal hormone depletion (Bastian et al., 2012; 2014; O'Shaughnessy et al., 2018; Royland et al., 2008). O'Shaughnessy et al (2018) demostrates dose-response relationships between cortical T4 and T3 concentrations and changes in a variety of neocortical genes (e.g., Parv, Col11a2, Hr, Ngf) that were "statistically significant at doses that decreased brain t4 and/or T3". There was no quantitation of this relationship reported.

In addition, there is very little known about whether compensatory processes are available in the developing hippocampus that may modulate the impact of serum levels on hippocampal gene transcription. These available data suggest that a 40-50% decrement in serum T4 in the pup, is sufficient to observe changes in hippocampal gene expression. This is similar to finding for loss of hearing function in rats following postnatal chemical-induced hypothyroxinemia (Crofton, 2004).

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Relationship: 1388: T4 in serum, Decreased leads to Hippocampal anatomy, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Most of the available data has come from rodent models. Human clinincal studies have documented changes in hippocampal volume in children with congenital hypothyroidism (Wheeler et al., 2011).

Key Event Relationship Description

The vast majority of brain thyroxine (T4) is from the serum. Once taken up from the serum, T4 is converted to triiodothyronine (T3) which binds to the nuclear receptors (TRα and TRβ) to control thyroid-mediated gene expression (Oppenheimer, 1983). It is well established that TH regulates genes critical for brain development (Bernal, 2007; Anderson et al., 2003). As such, the structural development of the hippocampus is modulated by TR-mediated gene transcription, and alterations in serum TH can adversely impact hippocampal neuroanatomy.

Evidence Supporting this KER

The weight of evidence for this indirect relationship is strong. There is a vast amount of literature that supports this KER in multiple species.

Biological Plausibility

The biological plausibility of this KER is rated as strong. The relationship is consistent with the known biology of the regulation of serum TH concentrations, brain TH concentrations, and the known action of TH to modulate genes critical for developmental processes that control structural development of the brain in general, including the hippocampus.

Empirical Evidence

The empirical support for this KER is strong. In humans, untreated congenital hypothyroidism and severe iodine deficiency are accompanied by reductions in circulating levels of TH, and result in severe structural alterations in brain size, including hippocampus (Wheeler et al., 2011). The tie to serum TH has been amply demonstrated in clinical therapy of hypothyroidism during pregnancy and in congenitaly hypothyroid children born to euthroid mothers. In addition, there is a vast amount of data from animal studies that support this relationship. Gross structural changes in the hippocampus following severe TH insufficiency are widely reported (Hasegawa et al., 2010; Powell et al.,2010; Madiera et al., 1991; 1992; Rami et al. 1986a 1986b; Madeira and Paula-Barbosa 1993; Rabie et al., 1980; Berbel et al., 1996). Other studies reveal more subtle changes in hippocampal structure such as reductions in a specific subregions of the hippocampus or of a cell type (eg. parvalbumin expressing inhibitory neurons) or synaptic component (ie synapsin, postsynaptic density proteins) or misplacement of cells within the hippocampal cell layers (Berbel et al., 1997; 2010; Gilbert et al., 2007; Auso et al., 2003; Gilbert et al., 2016; Cattani et al., 2013). These observations at the histological level are correlated with reductions in serum T4. The most profound structural impairments are typically seen with severe reductions in both hormones.

Additional evidence for a relationship between serum TH and hippocampal anatomy comes from the study of adult neurogenesis. The propensity of the hippocampus to generate new neurons throughout the lifetime of the organism occurs in only two brain regions, the olfactory bulb and the hippocampus. Severe reductions in circulating levels of TH in adulthood reduces both neuroprogenitor cell proliferation and survival of newly generated neurons in the neurogenic niche of the hippocampal dentate gyrus (Ambrogini et al., 2005, Montero-Pedrazuela et al., 2006, Kapoor et al., 2015). These same effects on neurogenesis also occur during development. However, the impact of developmental TH disruption on neurogenesis in adult offspring shows that the developing brain is more sensitive to these persistent effects. For example, a reduction in the capacity for neurogenesis was recently demonstrated in adult euthyroid offspring of developmentally TH compromised dams (Gilbert et al., 2016). These data indicate a permanent deficit in the capacity for neurogenesis, a process that controls dentate gyrus volume and cell number, following moderate reductions in serum TH in the fetus/neonate.

Finally, from in vitro studies, T3 stimulation accelerates the formation of GABAergic boutons and alters the distribution of GABAergic axons among growing neurons in culture. This growth is dependent on both activity within the network and the presence of T3. It can be blocked by the T3 nuclear receptor antagonist, 1-850, or pharmacological block of synaptic activity (Westerholz et al., 2010; 2013). T3 is believed to have this effect by it action on synaptic pruning. This example reveals the dynamic interplay between synaptic activity and neuroanatomy in the developing nervous system (Kozorovitskiy 2012).

> *Temporal Evidence:* The temporal nature of this KER is developmental (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered hippocampal anatomy. Reductions in serum TH in the neonate produced alterations in hippocampal parvalbumin-expressing neurons while the same treatment in adulthood is without effect (Gilbert et al., 2007). In a rodent model of prenatal TH deficiency, decreased length and number of radial glial cells which are critical for neuronal migration was reversed by hormone replacement treatment to the dam (Pathak et al., 2011). Reversibility of cortical layering defects with thyroxine treatment have also been reported in models of maternal hypothyroidism (Pathak et al., 2011; Berbel et al., 2010; Mohan et al., 2012). In in vitro studies, temporal specificity of the influence of T3 on GABAergic synapses and synaptic pruning has also been demonstrated (Westerholz et al., 2013). In addition, clinical therapy of hypothyroidism during pregnancy, and in congenitally hypothyroid

children born to euthroid mothers ameliorates most of the adverse impacts on the developing human brain.

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 developmental exposure to the PTU.

Uncertainties and Inconsistencies

This has been repeatedly demonstrated. However, with some studies noted above, most investigations have been conducted in the neonate after severe hormone reductions induced by PTU, MMI or thyroidectomy. These severe changes alter a wide variety of general growth and developmental processes. In one of the few dose-response studies assessing hippocampal anatomy, alterations in simple guidenline 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. More data is needed that link more limited decrements in serum TH to specific hippocampal anatomical changes. 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.

Quantitative Understanding of the Linkage

Response-response relationship

Most investigations for hippocampal anatomy have been conducted in the neonate after severe hormone reductions. There is currently insufficient data for quantitative analysis of serum T4 and hippocampal neuroanatomy.

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Relationship: 1389: T4 in serum, Decreased leads to Hippocampal Physiology, Altered

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Most of the data to support this KER are derived from rodent studies.

Key Event Relationship Description

Thyroid hormones are critical for normal development of the structure and function of the brain, including the hippocampus (Anderson et al., 2003; Bernal, 2007). Brain concentrations of T4 are dependent on transport of primarily T4 from serum, with subsequent conversion to T3 in the astrocytes by deiodinase and transfer to nuclear receptors within the neuron. This is followed by TH dependent gene transcription that influences hippocampal structural development and subsequent physiological function.

Evidence Supporting this KER

The weight of evidence for this indirect relationship is moderate. A wide variety studies have been performed in several labs in which thyroid hormone reductions in serum induced by chemicals/treatments, acting at a variety of target sites to disrupt hormonal status, is coincident with altered hippocampal physiology and/or plasticity. These include inhibition of TPO, NIS, dietary insufficiencies of iodine, and upregulation of liver catabolism, NIS inhibition, or dietary manipulation of iodine. Most of the data available is from the model TPO inhibitors, PTU and MMI, and this data documents enduring hippocampal physiological impairments in adult offspring following a period of transient serum TH insufficiencies in the pre- and post-natal period. Serum hormones are reported for the neonate and the dam at the termination of exposure, and recovery of hormonal status in the adult has been demonstrated in a number of studies despite the persistence of the hippocampal deficit. A few laboratories have reported dose-dependent effects at less than maximal hormone depletion.

Biological Plausibility

The biological plausibility of this KER is rated as strong. The relationship is consistent with the known biology of how TH control development of hippocampal physiology.

Empirical Evidence

Empirical support for this indirect KER is rated as strong. Empirical data from studies that measure serum TH concentrations and then assess alterations in synaptic function in the hippocampus have come from several laboratories. This work has employed in vivo, ex vivo and in vitro preparations from developmentally exposed animals.

Most of the in vivo neurophysiological assessments have been performed in the dentate gyrus. Excitatory and inhibitory synaptic transmission were reduced by PTU in a dose-dependent fashion (Gilbert and Sui, 2006; Gilbert et al., 2007; Gilbert, 2011). Serum T4 decrements in dams and pups were positively correlated with the synaptic impairments. Serum T4 and hippocampal excitatory transmission were also reduced in pups from dams exposed to perchlorate (Gilbert et al., 2008) and iodine deficiency (Gilbert et al., 2013). However, serum T4 reductions induced by the complex PCB mixture, A1254, were associated with increases not decrements in excitatory response amplitudes (Gilbert et al., 2003).

Impaired synaptic transmission and plasticity in the form of long-term potentiation (LTP) and long-term depression (LTD) have been reported using in vitro and ex vivo preparations (Sui and Gilbert, 2003; Sui et al., 2005; 2007; Gilbert and Sui, 2006; Gilbert and Paczkowski, 2003; Gilbert, 2011; Taylor et al., 2008; Vara et al., 2002), Dong et al., 2005; Gilbert, 2003; 2004; 2011; Gilbert et al., 2016;), .

In many studies these observations have been reported under conditions of severe hypothyroidism induced primarily by TPO-inhibitors MMI and PTU or severe iodine deficiency (Vara et al., 2002; Dong et al., 2005). In others, researchers produced graded degrees of TH insufficiency in dams and pups by administering varying doses of PTU, perchlorate, or dietary iodine deficiency, and

reported dose-dependency of the observed effects. This work has provided increased confidence in the relationship between TH insufficiency and functional impairment of the hippocampus, and the specificity of the observed effects to be mediated by TH insufficiency (Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2013; 2016).

As described in the KER entitled “Hippocampal anatomy, altered leads to Hippocampal Physiology, Altered”, there is dynamic reciprocal interplay between neuroanatomy and physiology, particularly evident in the developing nervous sytem, making it difficult to parse the effects of one independently of the other (Kozorovitsky et al., 2012). In the *in vitro* studies of Westerholz et al (2010; 2013), T3-induced increase in GABAgeric synapses is activity-dependent, in that the anatomical changes described required both spontaneous electical activity in the network in addition to thyroid hormone. The electrophysiological competence of that emerging synaptic network was similarly dependent upon the hormone stimulation in addition to the growth of the GABAergic neurons. In this manner, TH can directly influence the formation of emerging cortical networks. Although demontrated using cortical neurons, it is expected that very similar processes occur in the developing neural networks of the hippocampus.

> *Temporal Evidence:* The temporal nature of this KER is developmental (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered physiological function in the dentate gyrus (Gilbert 2011; Sanchez-Huerta et al., 2015). Rescue experiments have not been performed in developmental hypothyroid models. In *in vitro* studies, temporal specificity of the influence of T3 on network activity has been demonstrated (Westerholz et al., 2013).
>
> *Dose-Response Evidence:* There are several reports on the dose-dependent nature of the correlation between serum THs and changes in hippocampal physiology albeit from a limited number of laboratories (Taylor et al., 2008; Gilbert et al., 2007; 2016; Gilbert 2011; Gilbert and Sui, 2006).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some remaining uncertainties. It is widely accepted that changes in serum THs during development will result in alterations in behavior controlled by the hippocampus. This has been repeatedly demonstrated in animal models and in humans. However, most studies have been performed under conditions of severe hypothyroidism induced primarily by TPO-inhibitors MMI and PTU, or severe iodine deficiency. In addition, it is also known that there is an interaction between physiological and anatomical development, where anatomy develops first, and can be ‘reshaped’ by the ongoing maturation of physiological function (e.g., Kutsarova et al., 2017).

Quantitative Understanding of the Linkage

Response-response relationship

Insufficient data exist to date that could be used to develop a quantitative predictive model of neurophysiological in hippocampus from serum TH concentrations. The dynamic range over which neurophysiological endpoints can vary is small complicating the development of quantitative relationships between degree of TH insufficiency and magnitude of neurophysiological impairment.

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Relationship: 403: T4 in serum, Decreased leads to Cognitive Function, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

There is a plethora of data supporting this KER in rats, mice, and humans.

Key Event Relationship Description

Thyroid hormones (TH) are critical for normal development of the structure and function of the brain, including hippocampal development and cognitive function (Anderson et al., 2003; Bernal, 2007; Willoughby et al., 2014). Brain concentrations of T4 are dependent on transfer of T4 from serum, through the vascular endothelia, into astrocytes. In astrocytes, T4 is converted to T3 by deiodinase and subsequently transferred to neurons cellular membrane transporters. In the brain T3 controls transcription and translation of genes responsible for normal hippocampal structural and functional development. 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. Thus, there is an indisputable indirect link between serum T4 and cognitive function.

Evidence Supporting this KER

The weight of evidence for this indirect relationship is strong. Alterations in serum TH concentrations are very well correlated with adverse impacts on cognitive behaviors such as learning and memory. This includes a large amount of literature, from more than four decades of research, that links hypothyroidism and/or hypothyroxinemia with alterations in spatial cognitive function, a hippocampal dependent behavior. A number of reviews are cited below that are primarily from humans and rodents, but this indirect relationship has also been shown for a number of other species.

In humans, severe serum TH reductions that accompany congenital hypothyroidism dramatically impair brain function and lead to severe mental retardation. Lower global IQ scores, language delays and weak verbal skills, motor weakness, attentional deficits and learning impairments accompany low serum TH in children (Derksen-Lubsen and Verkerk 1996). Standard tests of IQ function in children born to mothers with even marginal hypothyoidism during pregnancy or in children with a defective thyroid gland who are then treated remain approximately 6 points below expected values. Selective deficits on visual spatial, motor, language, memory and attention tests are observed, the exact phenotype largely dependent on the developmental window over which the insufficiency occurred and the severity of the hormone deficit (Mirabella et al. 2000; Rovet 2002; Zoeller and Rovet 2004; Willoughby et al 2014). Indeed, this link is recognized as being so clinically important that T4 and TSH are monitored in all newborns in the US.

In rodent models, reductions in serum TH induced by TPO inhibitors such as MMI and PTU, when induced during development, lead to a variety of neurobehavioral impairments. These impairments can occur in the sensory, motor, and cognitive domains. The specific phenotype is dependent on both the window of exposure, the duration of exposure, and the severity of the hormone reduction (Zoeller and Rovet, 2004). This includes more than four decades of work linking serum TH changes to alterations in hippocampal-dependent spatial behaviors (Akaike et al., 2004; Axelstand etal., 2008; Brosvic et al; Kawada et al, 1988; Friedhoff et al, 2000; Gilbert and Sui, 2006; Gilbert et al., 2016; Gilbert, 2011).

Biological Plausibility

The biological plausibility of this KER is rated as strong. The relationship is consistent with the known biology of how the relationship between serum TH concentrations, brain TH concentrations, and TH control of brain development.

Empirical Evidence

Empirical support for this KER is rated as strong. Empirical data from studies that measure serum TH concentrations and then assess alterations in cognitive function, including hippocampal dependent behaviors, is vast. The qualitative relationship between reduced serum hormone levels and adverse cognitive outcomes is well accepted in endocrinology, as well as developmental neuroendocrinology. Indeed, the relationship between serum T4 and T3 levels and adverse neurodevelopmental outcomes (e.g., IQ loss in children) is beyond reproach.

Temporal Evidence: The temporal nature of this KER is developmental (Seed et al., 2005). It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in cognitive function. In humans, hormone insufficiency that occurs in mid-pregnancy due to maternal drops in serum hormone, and that which occurs in late pregnancy due to disruptions in the fetal thyroid gland lead to different patterns of cognitive impairment (Zoeller and Rovet, 2004). In animal models, deficits in hippocampal-dependent cognitive tasks result from developmental, but not adult hormone deprivation (Gilbert and Sui, 2006; Gilbert et al., 2016; Axelstad et al, 2009; Gilbert, 2011; Opazo et al., 2008). Replacement studies have demonstrated that varying adverse neurobehavioral outcomes, including cognitive function, can be reduced or eliminated if T4 (and/or T3) treatment is given during the critical windows (e.g., Kawada et al., 1988; Goldey and Crofton, 1998; Reid et al., 2007).

Dose-Response Evidence: An increasing amount of literature is now available that provides clear evidence of the 'dose-response' nature of this KER. Most research over that last 40 years has employed high doses of chemicals, or chemicals plus thyroidectomies, that results in severe depletion of circulating thyroid hormones. More recently, researchers produced graded degrees of TH insufficiency in dams and pups by administering varying doses of chemicals and have correlated them to the dose-dependency of the observed effects. This work has provided increased confidence in the relationship between serum TH decrements and a variety of neurodevelopmental impairments, and also to the specificity of the observed effects on brain

development that is directly mediated by TH insufficiency (Goldey et al., 1995; Crofton, 2004; Gilbert and Sui, 2006; Gilbert, 2011; Bastian et al., 2014; Royland et al., 2008; Sharlin et al., 2008).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some remaining uncertainties. It is widely accepted that changes in serum THs during development will result in alterations in behavior controlled by the hippocampus. This has been repeatedly demonstrated in animal models and in humans. A major uncertainty is the precise relationship between the degree, timing and duration of serum TH changes that leads to these behavioral deficits.

Inconsistencies may also exist for chemicals other than classical TPO inhibitors that may reduce serum TH and induce impairments in cognitive function, but through action on other endocrine systems, or via direct action on the brain in the absence of an intervening endocrine action.

Quantitative Understanding of the Linkage

Response-response relationship

Except for a quantitative relationship between serum T4 and hearing loss in rodents (Crofton, 2004), there are no other reports of development of quantitative predictive models linking serum TH and adverse neurological outcomes. Insufficient data exist to develop a quantitative predictive model of adverse cognitive outcomes from serum TH concentrations. However, evidence from human studies suggests that decreases as low as 25% in serum T4 in pregnant women will yield small decrements in IQ in children (e.g., Haddow et al., 1995). Since publication of this seminal paper, several reports have appeared providing supportive if not direct confirmatory data on the association of reductions in maternal or early postnatal serum TH and adverse neurodevelopmental outcomes (e.g., Rovet and Willoughby, 2010, Wheeler et al., 2011, Willoughby et al., 2014, Wheeler et al., 2015; Pop et al., 1999, Pop et al., 2003, Kooistra et al., 2006, Henrichs et al., 2010, Korevaar et al., 2016). Based on these data, regulatory authorities have used 10 and/or 20% changes in serum T4 as a point of departure for hazard assessments in rodent studies (EPA, 2011).

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