

Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with *In Vitro* Testing and Adverse Outcome Pathway Approaches

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BACKGROUND: Extensive clinical and experimental research documents the potential for chemical disruption of thyroid hormone (TH) signaling through multiple molecular targets. Perturbation of TH signaling can lead to abnormal brain development, cognitive impairments, and other adverse outcomes in humans and wildlife. To increase chemical safety screening efficiency and reduce vertebrate animal testing, *in vitro* assays that identify chemical interactions with molecular targets of the thyroid system have been developed and implemented.

OBJECTIVES: We present an adverse outcome pathway (AOP) network to link data derived from *in vitro* assays that measure chemical interactions with thyroid molecular targets to downstream events and adverse outcomes traditionally derived from *in vivo* testing. We examine the role of new *in vitro* technologies, in the context of the AOP network, in facilitating consideration of several important regulatory and biological challenges in characterizing chemicals that exert effects through a thyroid mechanism.

DISCUSSION: There is a substantial body of knowledge describing chemical effects on molecular and physiological regulation of TH signaling and associated adverse outcomes. Until recently, few alternative nonanimal assays were available to interrogate chemical effects on TH signaling. With the development of these new tools, screening large libraries of chemicals for interactions with molecular targets of the thyroid is now possible. Measuring early chemical interactions with targets in the thyroid pathway provides a means of linking adverse outcomes, which may be influenced by many biological processes, to a thyroid mechanism. However, the use of *in vitro* assays beyond chemical screening is complicated by continuing limits in our knowledge of TH signaling in important life stages and tissues, such as during fetal brain development. Nonetheless, the thyroid AOP network provides an ideal tool for defining causal linkages of a chemical exerting thyroid-dependent effects and identifying research needs to quantify these effects in support of regulatory decision making. <https://doi.org/10.1289/EHP5297>

Introduction

Regulatory programs within the U.S. Environmental Protection Agency (EPA) are responsible for protecting public health and the environment from the potential hazards and risks of chemical exposures. To quickly and economically predict chemical hazard, and reduce laboratory animal testing, the U.S. EPA and many other government institutions and stakeholders are placing an emphasis on designing, validating, and implementing alternative approaches to whole animal toxicity testing to support chemical safety assessments and risk-based decisions (ECHA 2016; ICCVAM 2018; National Academies of Sciences, Engineering, and Medicine 2007; U.S. EPA 2018). These alternative approaches, more recently termed new approach methodologies, may encompass any of a broad range of *in vitro* technologies [e.g., robotic-based higher-throughput screening (HTS), lower-throughput formats], omic approaches (e.g., microarray, RNA-sequencing, proteomics,

genome editing), and *in silico* modeling (e.g., molecular docking to model interactions of small molecules and proteins, computational read-across to predict the toxicity of data poor substances using data rich sources).

Coincident with the increasing availability of alternatives to traditional animal testing, government agencies have been seeking to adopt these new approach methodologies in chemical safety evaluations. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods recently finalized strategies for establishing new approaches in chemical safety evaluations for U.S. federal agencies (ICCVAM 2018). The U.S. EPA has published plans to promote the use of nonanimal alternative test strategies under the amended Toxic Substances Control Act (TSCA) (U.S. EPA 2018). Likewise, the U.S. EPA's Endocrine Disruptor Screening Program (EDSP), which screens and tests chemicals for interference with estrogen, androgen, and thyroid hormone (TH) signaling, has been transitioning toward use of *in vitro* HTS to evaluate chemicals for endocrine activity (U.S. EPA 2015b, 2017). *In vitro* HTS has been implemented under the EDSP to screen chemicals for interaction with estrogen and androgen receptors (Browne et al. 2015; Judson et al. 2015, 2017; Kleinstreuer et al. 2017; U.S. EPA 2014), as well as steroidogenesis pathways (Botteri Principato et al. 2018; Haggard et al. 2018a; Karmaus et al. 2016). Several *in vitro* HTS assays for identification of thyroid active chemicals are now available, or under development, and the ToxCast and Tox21 programs have implemented *in vitro* HTS for targets in the thyroid pathway (Collins et al. 2008; Dix et al. 2007; Judson et al. 2010; Kavlock et al. 2008; Thomas et al. 2018). In Europe, the Joint Research Commission European Union Reference Laboratory for Alternatives to Animal Testing has been developing and validating *in vitro* thyroid assays for test guidelines

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(EU Science Hub 2017), following an Organisation for Economic Co-operation and Development (OECD) scoping document on the availability of *in vitro* methods to investigate modulators of TH signaling (OECD 2014). Efforts to develop new methods for evaluating chemical interactions with the thyroid pathway have been informed by recent initiatives to evaluate the empirical evidence of chemical mechanisms of thyroid disruption (e.g., EU 2017; Murk et al. 2013).

To meet regulatory requirements for identifying potential endocrine disruptors, integration of a chemical's toxicity mechanisms and apical effects is needed. To date, this has been challenging for identifying chemicals that perturb TH signaling due to the limited availability of mechanistic and thyroid-relevant end points used to evaluate chemical safety. The OECD's recently updated Guidance Document 150 includes a revised conceptual framework and associated *in vitro* and *in vivo* test guidelines for evaluating endocrine disruption, including guidelines that have been updated to add thyroid end points (OECD 2018j). The European Chemicals Agency has adopted OECD Guidance Document 150 in its regulatory guidance for evaluating biocides and pesticides for endocrine disruption (Andersson et al. 2018).

Despite these recent advances, the application of new technologies to identify chemicals that exert effects through a thyroid mechanism continues to be complicated by a lack of linkages between chemical effects detected at macromolecular levels of biological organization (e.g., protein, cellular) and end points (e.g., serum TH) and adverse outcomes (e.g., developmental neurotoxicity) traditionally used in hazard assessment. Moreover, apical developmental end points collected in current regulatory test guidelines (Table 1) are not thyroid-specific and may occur through a variety of mechanisms, making it difficult to infer biological relevance, dose- and time-response relationships, and associated uncertainties. Taken together, regulatory test guidelines, when required for chemical assessments and regulatory decisions, continue to rely on thyroid measurements derived from *in vivo* animal testing with uncertainty about the mechanisms underlying adverse outcomes when observed.

Objectives and Overview of Challenges

To facilitate the regulatory application of data derived from these new technologies, we present an adverse outcome pathway (AOP) network (Figures 1–2) that links well-known and putative chemical targets of thyroid activity to downstream adverse outcomes. We examine current regulatory and scientific challenges in screening chemicals for thyroid-related perturbations, and propose how new *in vitro* technologies in the context of the thyroid AOP network can serve as a screening tool to identify chemicals that interact with targets in the thyroid pathway. Measuring chemical interactions with thyroid-related molecular targets can help elucidate whether adverse outcomes are mediated through a TH signaling mechanism.

TH signaling involves feedback interactions of the hypothalamic–pituitary–thyroid (HPT) axis, circulatory system, liver, and other tissues, collectively referred to here as the thyroid axis, that are critical to the maintenance of homeostasis and regulation of development and physiological functions (Bernal 2005; Denver 1998; Dussault and Ruel 1987; Oppenheimer and Schwartz 1997; Power et al. 2001; Silva 1995; Yen 2001; Zhang and Lazar 2000; Zoeller et al. 2005). In contrast to other endocrine pathways, evaluation of chemical impacts on the thyroid axis requires a more involved screening strategy because chemicals perturb TH signaling through several mechanisms other than the thyroid hormone receptor (TR) (Brucker-Davis 1998; Capen and Martin 1989; Capen 1997; DeVito et al. 1999; Hurley 1998; U.S. EPA 2015a). Clinical and experimental evidence describe

receptor-independent disruption of TH signaling that can result in reduced serum and tissue concentrations of TH that can lead to abnormal brain development, irreversible cognitive deficits, and other impairments, depending on the severity and timing of the TH insufficiency (as reviewed by Brucker-Davis 1998; Capen and Martin 1989; Crofton 2008; Diamanti-Kandarakis et al. 2009; Gilbert and Zoeller 2010; Gore et al. 2015; Hurley 1998; Köhrle 2008; Murk et al. 2013; Zoeller 2005).

To help link molecular targets that may mediate thyroid-dependent apical effects, AOPs can be integrated in an AOP network (Figure 1) that includes multiple molecular initiating events (MIEs) sharing one or more key events (KEs) leading to one or more adverse outcomes (Ankley et al. 2010; Browne et al. 2017; Paul Friedman et al. 2016; Villeneuve et al. 2014; Villeneuve et al. 2018; Wittwehr et al. 2017). The thyroid AOP network herein (Figure 2) provides a tool for mapping TH signaling data collected at multiple levels of biological organization and using differing study designs. It is intended to aid in evidence integration and determining if a chemical is exerting an adverse outcome by a thyroid mechanism.

Discussion

In Vitro HTS Assays Aimed at MIEs in the Thyroid Axis

Over two dozen MIEs in the thyroid axis are demonstrated or hypothesized to be potential targets of chemicals (Table 2). These MIEs have been described at length in a recent review (OECD 2014) and workshop (Murk et al. 2013), so they will only be briefly summarized here. As a result of these and other collaborative efforts over the years, an increasing number of *in vitro* HTS assays and lower-/medium-throughput assays amenable to high-throughput platforms have been developed to measure chemical interactions with MIEs that may lead to perturbed TH synthesis, delivery, metabolism, and signaling. Perhaps two of the most well-characterized MIEs in the thyroid axis involve disruption of TH production by chemical interference with iodide uptake into thyroid follicular cells by inhibition of the Na⁺/I[−] symporter (NIS) (Clewett et al. 2004; Lecat-Guillet et al. 2008; Richards and Ingbar 1959; Tietge et al. 2005, 2010; Wolff 1998; Wu et al. 2016) and by inhibition of thyroperoxidase (TPO) activity (Coady et al. 2010; Davidson et al. 1978; Degitz et al. 2005; Francis and Rennert 1980; Tietge et al. 2010). An *in vitro* radioactive iodide uptake assay coupled to a human NIS-expressing HEK293T cell line has been developed and adapted for use as an *in vitro* HTS assay to identify chemicals that may inhibit iodide uptake by NIS (Hallinger et al. 2017; Wang et al. 2018). Another *in vitro* HTS assay has been developed to use the commercially available fluorescent peroxidase substrate, Amplex UltraRed to detect TPO inhibition (the “AUR-TPO” assay), and was used to screen approximately 1,000 chemicals in the ToxCast phase 1 and 2 libraries (Paul Friedman et al. 2016; Paul et al. 2014).

Extrathyroidal targets involved in chemical perturbations of TH metabolism and transport have also been the focus of *in vitro* HTS development. Some chemicals have been shown to inhibit the activity of iodothyronine deiodinase (DIO) enzymes in different species, tissues, and life stages (Butt et al. 2011; Capen and Martin 1989; Ferreira et al. 2002; Hood and Klaassen 2000; Morse et al. 1993; Noyes et al. 2011, 2013). The three DIO isoforms, Types I, II, and III (DIO1, DIO2, and DIO3, respectively) are differentially expressed and critical to the maintenance of TH in circulation and target tissues, such as brain (Dentice et al. 2013; Gereben et al. 2008; Köhrle 1999; Salvatore et al. 1996; St Germain et al. 1994). A low-throughput colorimetric assay (Renko et al. 2012) has been adapted recently to 96-well plate

Table 1. U.S. EPA and OECD test guidelines with required (X) and optional (opt) *in vivo* thyroid end points and potentially thyroid-responsive adverse outcomes; laid out according to the adverse outcome pathway (AOP) diagram in Figure 1.

Test guideline	Study title	Life stage	TSH	T4	T3	Thyroid weight	Thyroid histopathology	Potential thyroid-responsive end points	Potential thyroid-responsive adverse outcomes	References
Mammalian (rat) models										
OCSPSP 890.1450/ 890.1500	Pubertal development (EDSP Tier 1)	Peripubertal	X	X	—	X	X	—	—	U.S. EPA 2009
OCSPSP 890.1400, OECD TG 441 ^a	Hershberger (EDSP Tier 1)	Castrated-peripubertal	—	opt	opt	—	—	—	—	OECD 2009; U.S. EPA 2009
OCSPSP 870.3050, OECD TG 407	28-d oral toxicity ^{b,c}	Young adult	opt	opt	—	opt	X	—	—	OECD 2018g; U.S. EPA 2000a
OCSPSP 870.3100, OECD TG 408	90-d oral toxicity ^{b,c}	Young adult	X	X	X	X	X	—	—	OECD 2018h; U.S. EPA 1998a
OCSPSP non-guideline OCSPSP 870.3700, OECD TG 414	CDT assay ^d Prenatal developmental toxicity ^e	Dam, offspring Dam, offspring	F0 (♀), F1 F0 (♀)	F0 (♀), F1 F0 (♀)	F0 (♀), F1 F0 (♀)	F0 (♀), F1 F0 (♀)	F0 (♀), F1 F0 (♀)	External, soft tissue, and skeletal malformations	Altered locomotor and sensory functioning in offspring (OECD only)	U.S. EPA 2005 OECD 2018f; U.S. EPA 1998b
OCSPSP 870.3650, OECD TG 421/422	Combined 28-d, repro/developmental toxicity ^c	Parent, offspring	opt	F0 (♂), F1 & F0 (♀): “if relevant”	—	F0: opt, F1: X	F0: opt, F1: X	Gross developmental malformations	Altered locomotor and sensory functioning in offspring	OECD 2018i; U.S. EPA 2000b
OCSPSP 870.3800, OECD TG 443/416	EOGRT/two-gen reproduction ^c	Parent, offspring	F0, F1	F0, F1	—	F0, F1	F0, F1	Brain, CNS, and PNS histology and morphology, startle response habituation	Altered locomotor and sensory functioning in offspring	OECD 2018d, 2018k
OCSPSP 870.6300, OECD TG 426	Developmental neurotoxicity	Parent, offspring	—	—	—	—	—	Brain, CNS, PNS histology and morphology, startle response habituation, learning, and memory	Altered locomotor, sensory, and cognitive functioning in offspring	OECD 2018c; U.S. EPA 1998c
OCSPSP 870.4100-4300, OECD TG 451-453	Chronic toxicity/carcinogenicity	Young adult	—	—	—	X	X	Thyroid gland hypertrophy and hyperplasia	Thyroid tumors (rodent)	OECD 2018b; U.S. EPA 1998d
Non-mammalian animal models										
OCSPSP 890.1100, OECD TG 231 ^a	AMA (EDSP Tier 1)	Metamorphosis	—	—	—	—	X	Growth (e.g., BW, HLL, SVL), developmental progression	Reduced survival, arrested or impaired metamorphosis	OECD 2018a; U.S. EPA 2009
OCSPSP 890.2300, OECD TG 241 ^a	LAGDA (EDSP Tier 2)	Metamorphosis	—	—	—	—	X	Growth (e.g., BW, SVL), developmental progression	Reduced survival, arrested or impaired metamorphosis	OECD 2018e; U.S. EPA 2009
OCSPSP 890.2100	Avian two-gen toxicity test (EDSP Tier 2)	Parent, offspring	X	X	—	X	X	Embryonic, hatching, and chick malformations	Reduced survival, impaired hatching, chick development	U.S. EPA 2009

Note: *In vivo* data collected under regulatory test guidelines can be mapped along an adverse outcome pathway (AOP) from early key events that indicate thyroid activity with high specificity to downstream biological responses and adverse outcomes that may or may not occur via a thyroid mechanism. Currently, no *in vitro* regulatory guidelines test chemical interactions with molecular targets in thyroid axis. —, Not applicable; AMA, amphibian metamorphosis assay; BW, body weight; CDT, comparative developmental thyroid; CNS, central nervous system; EDSP, Endocrine Disruptor Screening Program; EOGRT, extended one-generation reproduction test; F0, parental generation; F1, offspring, first generation; HLL, hind leg length; LAGDA, larval amphibian growth and development assay; OCSPSP, Office of Chemical Safety and Pollution Prevention; OECD, Organisation for Economic Co-operation and Development; opt, optional end point; PNS, peripheral nervous system; SVL, snout vent length; TG, test guideline; TSH, thyroid stimulating hormone; X, required end point.

^aU.S. EPA and OECD test guidelines harmonized with one another to minimize duplications and variations in test methodologies.

^bThe 90-d and 28-d repeated-dose toxicity studies include additional test methodologies for inhalation and dermal dosing of test animals, depending on observed or predicted routes of exposure.

^cUnder U.S. EPA test guidelines, specific hormone measures in serum are not required (OCSPSP 870.3800, 3700) or recommended for consideration if the test chemical is known or suspected to have an effect (OCSPSP 870.3050, 3100, 3650).

^dCDT (comparative developmental thyroid) assay in rats is a non-guideline study that has been required by the U.S. EPA's Office of Pesticide Programs to supplement reproductive toxicity testing with thyroid-related data in pregnant and nursing dams, their fetuses, and offspring (U.S. EPA 2005).

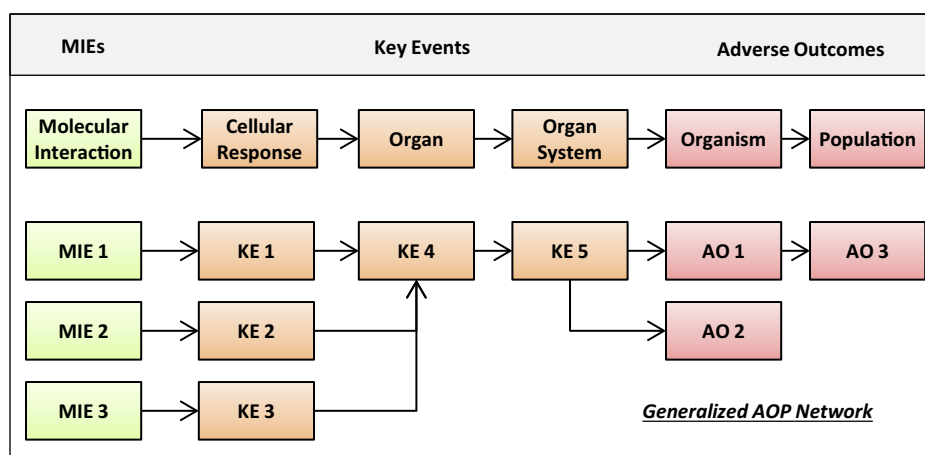


Figure 1. An adverse outcome pathway (AOP) begins with a molecular initiating event (MIE) and terminates in an adverse outcome that is linked by a series of intermediate key events (KEs) at increasing levels of biological organization. Adverse outcomes at the organism level are used in human health risk assessment and typically with plausible linkages to the population level for ecological risk assessment. A simplified example of an AOP network is presented whereby three MIEs shown in the first column (shaded green) elicit specific cellular responses in the next three columns (KE1, KE2, KE3; shaded orange) that converge in shared organ pathology (KE4) and mediate downstream organ system alterations (KE5) to produce divergent adverse outcomes at the organism (AO1, AO2) and population (AO3) levels as shown in the last two columns (shaded red).

format using an adenovirus that expresses human DIO1, and was optimized to detect DIO1 inhibition by chemicals in the ToxCast phase 1 library (Hornung et al. 2018). This assay is currently being used to design *in vitro* HTS assays to evaluate chemical inhibition of DIO2 and DIO3 (Olker et al. 2019). Chemicals also may act as ligands that can bind TH distributor proteins, notably transthyretin (TTR), and to a lesser extent thyroid binding globulin (TBG), in serum and cerebrospinal fluid (CSF) to displace native thyroxine (T4) (Brouwer et al. 1986; Cheek et al. 1999; Ishihara et al. 2003; Meerts et al. 2000; Ren and Guo 2012; Weiss et al. 2009). Although the overall *in vivo* relevance of chemical interference with serum TH distributors remains unclear, a surface plasmon resonance–based biosensor assay for TTR and TBG is available that provides medium- to high-throughput testing capabilities with commercially available technologies (Marchesini et al. 2006, 2008).

Another mechanism by which chemicals decrease circulating concentrations of TH is by activation of hepatic xenobiotic nuclear receptors (NRs) leading to inductions of phase I, II, and III metabolic enzymes and transporters in the liver and other tissues. Enhanced phase II TH glucuronidation and sulfation catalyzed by uridine diphosphate glucuronosyltransferases (UDPGTs) and sulfo-transferases (SULTs), respectively, can increase TH catabolism and reduce serum TH by accelerating clearance (Barter and Klaassen 1994; Hood et al. 2003; Yu et al. 2009; Zhou et al. 2002). *In vitro* HTS assays are available in ToxCast to assess chemical binding and activation of specific xenobiotic NRs [e.g., constitutive androstane receptor (CAR); pregnane X receptor (PXR); aryl hydrocarbon receptor (AhR)]. Once bound, activation of these NRs may up-regulate expression of phase I (CYP450s) and phase II [e.g., UDP glucuronosyltransferase family 1 member A1 and member A6 (*UGT1A1* and *UGT1A6*, respectively), Sulfotransferase family 2A member 1 (*SULT2A1*)] genes encoding isoenzymes involved in T4 glucuronidation and sulfation. *In vitro* HTS assays measuring induction and/or inhibition of TH conjugating enzymes, as well as the transporters mediating cellular transport of TH, are under development.

With regard to receptor–ligand interactions, screening of the Tox21 chemical library of 10,000 chemicals using transactivation assays aimed at TR α and TR β indicate that binding is restricted to a limited number of chemicals (Freitas et al. 2011, 2014; Houck et al. 2018; U.S. EPA 2015a). Likewise, chemical interactions with the thyrotropin releasing hormone receptor (TRHR) and thyroid

stimulating hormone receptor (TSHR) do not appear to be prominent sites for chemical binding (Gershengorn and Neumann 2012; Murk et al. 2013); however, this is an area with limited research and analysis of the ToxCast/Tox21 screening data is underway (Paul Friedman et al. 2017; Shobair et al. 2019).

Thyroid AOP Network as Framework for Chemical Screening and Assessment

The thyroid AOP network in Figure 2 illustrates how *in vitro* HTS assays (under development or currently available) identified in Table 2 can be mapped to this network of KEs that chart a progressive path to adverse outcomes. The thyroid AOP network serves as the foundation to organize and evaluate thyroid data, identify data gaps, and examine the evidence for causality between KEs in AOPs. These KE relationships connecting MIEs to KEs and adverse outcomes in Figure 2 may be considered hypothesized, correlative, or causal depending on the strength of the evidence (e.g., Coady et al. 2017; Crofton and Zoeller 2005; Degitz et al. 2005; Hassan et al. 2017; Miller et al. 2009; Murk et al. 2013; Perkins et al. 2013; Zoeller and Crofton 2005).

Another important application of the thyroid AOP network is to clarify research needs for decision making. *In vitro* HTS assays currently available to measure MIEs are shown in Figure 2 with solid borders in the left-hand column (and also shaded green), and end points collected as part of U.S. EPA and OECD guidelines (Table 1) are shown with thick red borders. Several MIEs in Figure 2 and further summarized in Table 2 do not yet have *in vitro* HTS assays even though in some instances lower-throughput *in vitro* assays are available (e.g., for UDPGT/SULT activity). In addition, results from studies published in peer-reviewed literature may be another source of data to further populate the AOP network. The peer reviewed literature may be particularly helpful in identifying and characterizing KEs downstream of serum TH and proximal to adverse outcomes as current regulatory test guidelines (Table 1) provide limited coverage.

Advancements of methods for systematic review and evidence integration provide approaches to identify and evaluate peer-reviewed studies for relevance, performance, and reliability to support their inclusion in chemical assessment (Hoffmann et al. 2017; Rooney et al. 2014; National Academies of Sciences, Engineering, and Medicine 2018). An increasing number of omic studies also

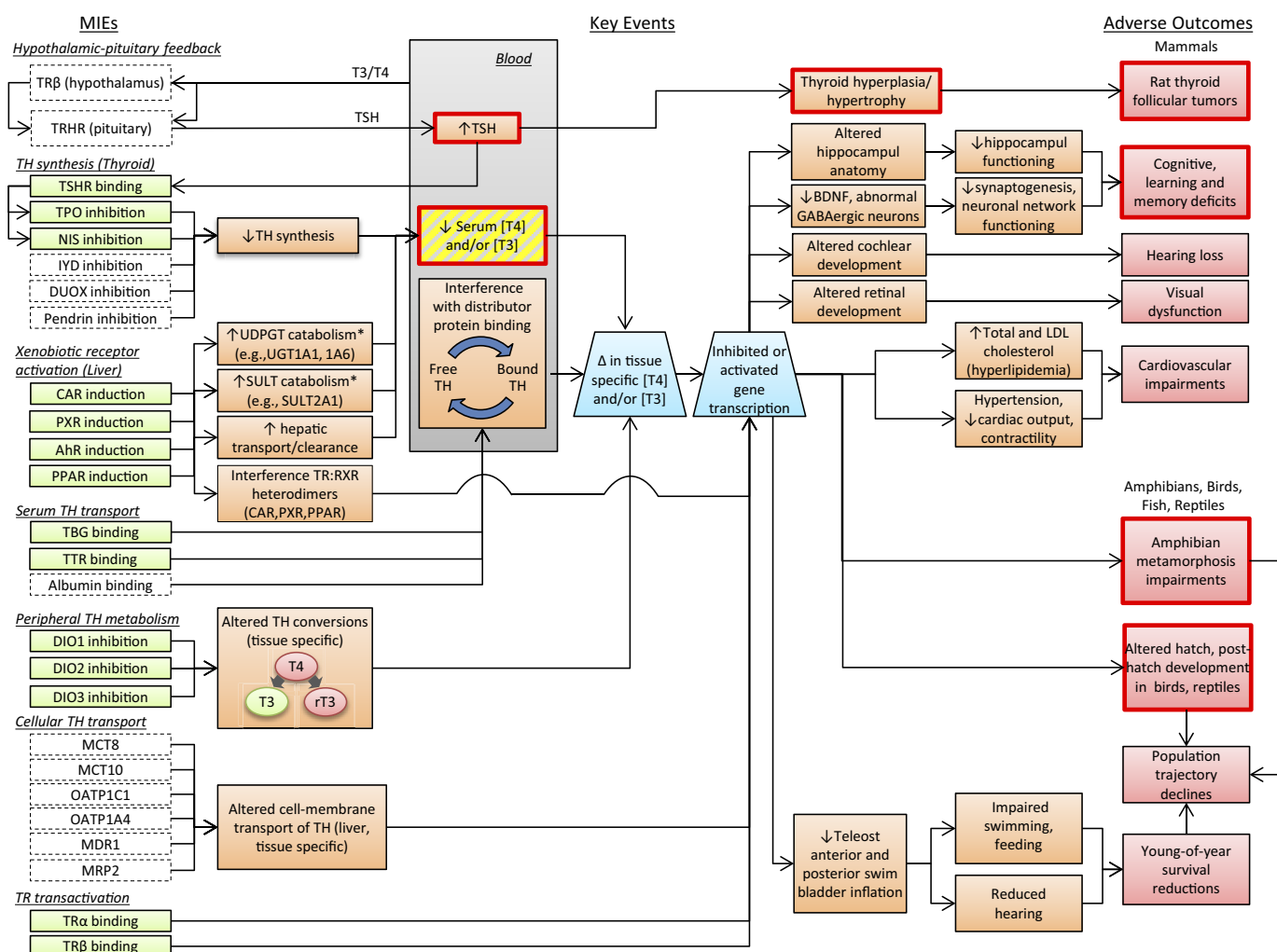


Figure 2. Adverse outcome pathway (AOP) network for chemically induced thyroid activity showing the integration of multiple individual AOPs under development and proposed. Biological linkages described may be informed by *in vitro*, *in vivo*, or computational data and may be causal, inferential, or putative, depending on the strength of the evidence. Boxes with thick, red borders represent *in vivo* end points that are targeted by U.S. EPA and OECD test guidelines. In the left-hand column, MIE boxes with solid borders (shaded green) represent current MIEs with *in vitro* high-throughput screening (HTS) assays that have demonstrated reliability and are available for use in thyroid activity screens, whereas those with dashed borders represent putative MIEs in the thyroid axis currently without *in vitro* HTS capabilities. In the key events (KEs) column, the box with the striped background (shaded yellow) depicts changes in serum TH as a KE node that represents a biomarker of thyroid disruption, whereas the trapezoids (shaded blue) represent additional potential KE nodes with limited data. Uppercase nomenclature denoting human protein is shown although present in differing species. Asterisks represent KEs being treated as MIEs. References and AOPs supporting the thyroid AOP network are identified in Table 2. AhR, aryl hydrocarbon receptor; BDNF, brain-derived neurotrophic factor; CAR, constitutive androstane receptor; DIO, iodothyronine deiodinase; DIO1, type 1 deiodinase; DIO2, type 2 deiodinase; DIO3, type 3 deiodinase; DUOX, dual oxidase; IYD, iodothyronine deiodinase; LDL, low-density lipoprotein; MDR, multidrug resistance protein; MCT, monocarboxylate transporter; NIS, sodium-iodide symporter; OATP, organic anion transporter polypeptide; OECD, Organisation for Economic Co-operation and Development; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; rT3, reverse T3 (3,3',5'-triiodothyronine); RXR, retinoid X receptor; SULT, sulfotransferase; T3, 3,3',5'-triiodothyronine; T4, thyroxine; TBG, thyroid binding globulin; TH, thyroid hormone; TPO, thyroperoxidase; TR, thyroid hormone receptor; TRHR, thyrotropin releasing hormone receptor; TSHR, thyroid stimulating hormone receptor; TTR, transthyretin; UDPGT, uridine diphosphate glucuronosyltransferase.

describe chemical effects on genes (transcriptomics), proteins (proteomics), and low-molecular-weight biomolecules (metabolomics) in the thyroid gland and TH-responsive tissues, including several transcriptomic studies (Boucher et al. 2014; Crump et al. 2002; Huang et al. 2011; Haggard et al. 2018b; Ohara et al. 2018; Porreca et al. 2016; Royland et al. 2008). For example, chromatin immunoprecipitation with microarray (ChIP-on-chip) and DNA sequencing (ChIP-seq) have been used in genome-wide analyses to identify putative TR target genes in human cancer cell lines (Chung et al. 2016), mouse liver (Grøntved et al. 2015; Ramadoss et al. 2014), mouse brain (Compe et al. 2007; Dong et al. 2009), and frog intestine (Fu et al. 2017). There are now a handful of studies reporting chemical effects on thyroid-related proteomics (e.g., Williams et al. 2016; Lee et al. 2018; Serrano et al. 2010) and

metabolomics (e.g., SSY Huang et al. 2016; Houten et al. 2016; Johnson et al. 2012). Cell-based assays and transgenic animal models have also been deployed to elucidate molecular targets, toxicity pathways, and chemical structure-activity relationships of thyroid disruption (Gentilcore et al. 2013; Jarque et al. 2018; Ji et al. 2012; Opitz et al. 2012; Rosenberg et al. 2017). To this end, recent advances in genome editing tools with toxicological applications, notably clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 technologies have been used to better understand TH signaling pathways (Kyono et al. 2016; Markossian et al. 2018; Sakane et al. 2018; Trubiroha et al. 2018; Yang et al. 2018). However, despite these advances, inclusion of *in vitro* genotyping studies in chemical assessment is still rare, and current efforts seek to standardize methods and link genetic outcomes to phenotypic

Table 2. Identification of known and putative molecular targets [i.e., molecular initiating events (MIEs) and key events (KEs) of chemical-induced thyroid disruption.

Molecular initiating event	Toxicological mechanism ^a	<i>In vitro</i> HTS assay readiness ^b	Potential adverse outcomes ^c	References
TH synthesis (thyroid gland) Sodium-iodide symporter (NIS)	Regulates serum iodide uptake into thyroid follicular cells and other tissues. Inhibition of NIS-iodide transport disrupts T4 and T3 synthesis. Well-characterized chemical target in thyroid pathway. <i>Chemicals: perchlorate, chlorate, nitrate, thiocyanate, small ion drugs</i>	<i>Existing:</i> Hallinger et al. 2017; Wang et al. 2018	Mammals: Developmental neurotoxicity, cognitive defects Amphibians: Impaired metamorphosis Birds: Delayed hatching, increased mortality, decreased growth <i>AOPs: 54, 110, 134, 176</i>	<i>In vivo</i> studies: Gilbert and Sui 2008; Goleman et al. 2002; McNabb et al. 2004; Tietge et al. 2005, 2010; York et al. 2004 <i>In vitro</i> studies: Chen et al. 2008 Reviews: NRC 2005
Thyroperoxidase (TPO)	Catalyzes oxidation of iodide, nonspecific iodination of tyrosyl residues of thyroglobulin (Tg), and coupling of iodotyrosyls to form Tg-bound T3 and T4. Inhibition of TPO activity disrupts TH synthesis. Well-characterized chemical target in thyroid pathway. <i>Chemicals: methimazole, PTU, propylthiouracil, isoflavones, mancozeb, resorcinol, triclosan</i>	<i>Existing:</i> Paul Friedman et al. 2016	Mammals: Visual deficits, developmental neurotoxicity, cognitive defects; MOA/AOP developmental neurotoxicity in rat Rat: Thyroid cancer Amphibians: Impaired metamorphosis Fish: Reduced swim bladder inflation <i>AOPs: 42, 119, 159, 175, 271</i>	<i>In vivo</i> studies: Ausó et al. 2004; Boyes et al. 2018; Degitz et al. 2005; Fort et al. 2000; Gilbert 2011; Gilbert et al. 2013, 2016; Goodman and Gilbert 2007; Lasley and Gilbert 2011; Nelson et al. 2016; O'Shaughnessy et al. 2018a, 2018b; Stinckens et al. 2016; Zoeller and Crofton 2005 <i>In vitro</i> studies: Davidson et al. 1978 Reviews: Dellarco et al. 2006; Hurley 1998 <i>In vivo</i> studies: Olker et al. 2018b <i>In vitro</i> studies: Shimizu et al. 2013
Iodotyrosine deiodinase (IYD)	Scavenges/recycles iodide in the thyroid by catalyzing deiodination to T1 and T2. Limited evidence of chemical inhibition. <i>Chemicals: 3-nitro-L-tyrosine, OH-PCBs, OH-PBDEs, rose bengal</i>	Promising	Amphibians: Impaired metamorphosis <i>AOP: 188</i>	—
Pendrin	Transports iodide from cytosol of thyroid follicular cell into lumen for organification. No reports of chemical interactions; research limited. <i>Chemicals: Unknown</i>	Early	Not yet characterized <i>AOP: 192</i>	—
Dual oxidase (DUOX)	Generates peroxide necessary for TH synthesis. No reports of chemical interactions. Research limited. <i>Chemicals: Unknown</i>	Early	Not yet characterized <i>AOP: 193</i>	—
TH transport (serum) Transferrin (TTR); Thyroid binding globulin (TBG); Albumin	Bind and distribute TH in circulation. TTR and TBG are known chemical targets. Albumin is the most abundant, but TH binding is nonspecific with low affinity. <i>Chemicals: OH-PCBs, OH-BDEs, PFAS, TBBPA, TCBCPA, genistein, dioxins</i>	<i>Existing:</i> Marchesini et al. 2006; Montano et al. 2012	Not yet characterized <i>AOP: 152</i>	<i>In vivo</i> studies: Hallgren and Darnierud 2002; Hedge et al. 2009
TH metabolism and excretion (liver and other target tissues) Iodothyronine deiodinase (DIO) Type 1 (DIO1); DIO	Control the activation and inactivation of T4 in a tissue-specific and temporal manner. With the exception of FD&C red dye no. 3 that has	<i>Existing:</i> DIO1: Hornung et al. 2018 DIO2, DIO3: Olker et al. 2019	Not yet characterized <i>AOPs: 156-158, 189-191</i>	<i>In vitro</i> studies: Cheek et al. 1999; Hamers et al. 2006; Lans et al. 1994 Reviews: Brouwer et al. 1998 <i>In vivo</i> studies: Borzelleca et al. 1987; Hood and Klaassen 2000; Mol et al. 1999;

Note: Table adapted from Murk et al. (2013) and OECD (2014). —, Not applicable; BPA, bisphenol A; EE2, 17 α -ethinylestradiol (synthetic estrogen); MOA, mode of action; NR, nuclear receptor; OH-BDE, hydroxylated bromodiphenyl ether (PBDE metabolites); OH-PCB, hydroxylated polychlorinated biphenyl ether (PCB metabolites); PAH, polycyclic aromatic hydrocarbon; PBDE, polychlorinated biphenyl ether; PCN, p-nonylphenol; PFAS, per- and polyfluoroalkyl substances; PTU, propylthiouracil; RXR, retinoid X receptor; T3, triiodothyronine; T4, thyroxine; TBBPA, tetrabromobisphenol A; TCBCPA, tetrachlorobisphenol A; TRIAC, triethoxyacetate acid; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; UDPGCT, uridine diphosphate glucuronosyltransferase.

^aThe toxicological mechanism column highlights the role of MIEs in thyroid hormone (TH) signaling along with chemicals shown to interact with them.

^b*In vitro* HTS assay development denoted as "promising" indicates MIEs in the thyroid axis for which there is interest and/or activity in developing *in vitro* HTS approaches, typically with supportive *in vivo* and slow- or medium-throughput *in vitro* toxicity studies indicating chemical interactions. *In vitro* HTS readiness denoted as "early" indicates putative MIEs but with limited toxicity evidence and with little current activity to develop high-throughput alternatives.

^cMIEs in the thyroid axis with evidence of linkages to adverse outcomes. Additional information on individual AOPs in development and completed can be found at <https://aopwiki.org/>.

Table 2. (Continued.)

Molecular initiating event	Toxicological mechanism ^a	<i>In vitro</i> HTS assay readiness ^b	Potential adverse outcomes ^c	References
Type 2 (DIO2); DIO Type 3 (DIO3)	been shown to induce thyroid tumors in rats, no studies to date have shown chemicals that exert effects on DIO expression and/or activity to directly manifest in adverse outcomes. <i>Chemicals: FD&C red dye no. 3, phenobarbital, PCN PTU, PCBs, PBDEs</i>			Morse et al. 1993; Noyes et al. 2011, 2013; Szabo et al. 2009 <i>In vitro</i> studies: Butt et al. 2011; Capen and Martin 1989; Ferreira et al. 2002; Olker et al. 2019; Renko et al. 2015
Constitutive androstane receptor (CAR); Pregnane X receptor (PXR); Aryl hydrocarbon receptor (AhR)	Xenobiotic nuclear receptors that up-regulate expression of phase I and II metabolic enzymes and phase III uptake and efflux transporters, some of which may accelerate TH catabolism and clearance. <i>Chemicals: See UDPGTs and SULTs</i>	<i>Existing:</i> ToxCast/Tox21 He et al. 2011; Maglich et al. 2003; Romanov et al. 2008; Rosenfeld et al. 2003	See UDPGTs and SULTs	See UDPGTs and SULTs
Uridine diphosphate glucuronosyltransferase (UDPGTs; e.g., UGT1A1, UGT1A6); sulfotransferases (SULTs; e.g., SULT2A1)	Major phase II chemical conjugation pathways that also regulate TH catabolism. Chemical up-regulation in the expression and activity of UDPGTs and SULTs increase T4 glucuronidation and sulfation, respectively. There are numerous isoforms of UDPGTs and SULTs, with UGT1A1, UGT1A6, and SULT2A1 having been shown to metabolize T4. <i>Chemicals: OH-BDEs, OH-PCBs, PAHs, PFAS, BPA, triclosan, dioxins, propiconazole, phthalates, phenobarbital, rifampicin, PCN</i>	Promising	UDPGTs: Mammalian cochlear damage and hearing loss; MOA/AOP hearing deficits via up-regulated TH catabolism. <i>AOPs 8, 194</i>	<i>In vivo</i> studies: Barter and Klaassen 1992, 1994; Haines et al. 2018; Klaassen and Hood 2001; Szabo et al. 2009; Vansell and Klaassen 2002; Visser et al. 1993; Wong et al. 2005; Yu et al. 2009
Alanine side-chain reactions	T4 and T3 alanine side-chains can be metabolized by oxidative decarboxylation or deamination, producing thyroamines and thyroacetic acids, respectively. <i>Chemicals: Unknown</i>	Early	SULTs: Not yet characterized Not yet characterized	<i>In vitro</i> studies: Butt and Stapleton 2013; Larson et al. 2011; Rotroff et al. 2010; Schuur et al. 1998 Reviews: Crofton and Zoeller 2005; Komno et al. 2008; Wang and James 2006 Reviews: Scanlan 2009; Wu et al. 2005
Peroxisome proliferator-activated receptor (PPAR α , PPAR β/δ , PPAR γ)	Key regulators controlling lipid and carbohydrate metabolism, as well as in mediating cellular differentiation and proliferation, and reproductive development. PPARs and TRs bind to DNA response elements as heterodimers with the RXR and other NRs and have been shown to compete for binding with RXR as well as for TR-transcriptional coactivators and corepressors. <i>Chemicals: PFAS, PBDEs, phthalates, BPA, TBBPA, TCBPA, organochlorine pesticides, EE2, fibrate drugs, Wy14,643, rosiglitazone, thiazolidinediones</i>	<i>Existing:</i> ToxCast/Tox21: R Huang et al. 2016; Martin et al. 2010; Romanov et al. 2008; van Raalte et al. 2004	Not yet characterized	<i>In vivo</i> studies: Lake et al. 2016; Springer et al. 2012 <i>In vitro</i> studies: Huang et al. 2011; Judge-Aubry et al. 1995 <i>In silico</i> studies: Nolte et al. 1998 Reviews: Hyyti and Portman 2006; Lu and Cheng 2010; Miller et al. 2009; White et al. 2011
TH transport (cellular) Monocarboxylate transporter (MCT8, MCT10); organic anion transporter polypeptide (e.g., OATP1C1; OATP1A4);	MCT8 is a specific cellular transporter of TH, and MCT8 mutations produce hypothyroidism and severe neurological impairments. MCT10, OATP1C1, and OATP1A4 mediate transport of TH and other ligands. There are numerous	Early	Not yet characterized	<i>In vivo</i> studies: Braun et al. 2012; Heuer et al. 2005; Noyes et al. 2013; Richardson et al. 2008; Roberts et al. 2008; Sharlin et al. 2018; Song et al. 2016; Westholm et al. 2009

Table 2. (Continued.)

Molecular initiating event	Toxicological mechanism ^a	<i>In vitro</i> HTS assay readiness ^b	Potential adverse outcomes ^c	References
Multidrug resistance protein (MDR1); multidrug resistance associated protein (MRP2)	other transporters that have been shown to transport TH, including several other subtypes of OATPs and L-type amino acid transporter (LAT1, LAT2). Limited evidence that some chemicals may alter expression. <i>Chemicals: tyrosine kinase inhibitors, fenamate drugs, TRIAC, PBDEs</i> Phase III hepatic efflux transporters that mediate hepatobiliary efflux of xenobiotics and TH. Importance as a chemical target is unclear. <i>Chemicals: PBDEs, anxiolytic/antileptic drugs</i>	Early	Not yet characterized	<i>In vitro</i> studies: Dong and Wade 2017; Friesema et al. 2003 Reviews: Visser et al. 2011
Receptor–ligand binding TRH receptor	Controls synthesis and release of TSH; TRH mutations lead to hypothyroidism. <i>Chemicals: Unknown</i> When activated, stimulates adenylyl cyclase and formation of cAMP that increases iodide uptake and TH synthesis in thyroid follicular cells. <i>Chemicals: Unknown</i>	Promising: ToxCast/Tox21	Not yet characterized	<i>In silico</i> studies: Engel et al. 2008; Knudsen et al. 2011; Sipes et al. 2013 Reviews: Beck-Peccoz et al. 2006 <i>In vitro</i> studies: Iomaa et al. 2013; Neumann et al. 2009; Santini et al. 2003; Titus et al. 2008 <i>In silico</i> studies: Paul Friedman et al. 2017; Shobair et al. 2019 Reviews: Gershengorn and Neumann 2012 <i>In vivo</i> studies: Dupré et al. 2004
TSH receptor	Transcription factors that have ligand (T3)-dependent and -independent activity. In humans, THRA genes encode TRα1, TRα2, and TRα3 and truncated isoforms. THRB genes encode TRβ1, TRβ2, and TRβ3 (possibly rat only) and truncated isoforms. Only TRα1, TRβ1, TRβ2, and TRβ3 can bind T3 and TREs; TRβ1 and TRβ2 regulate TRH in the hypothalamus. Some chemicals bind TRs as antagonists and/or modify transcription; however, screens of chemical libraries suggest binding is restricted. <i>Chemicals: TBBPA, TCBCA, BPA, OH-PCBs, OH-BDEs, triclosan</i>	Promising: ToxCast/Tox21	Not yet characterized	<i>In vitro</i> studies: Cheek et al. 1999; Clerget-Froidevaux et al. 2004; Freitas et al. 2011, 2014; Gauger et al. 2007; Hofmann et al. 2009; Huang et al. 2011; Kitamura et al. 2002, 2005; Kojima et al. 2009; Moriyama et al. 2002; Schriks et al. 2006; Sun et al. 2009; You et al. 2006 <i>In silico</i> studies: Knudsen et al. 2011; Politi et al. 2014; Romanov et al. 2008; Sipes et al. 2013 Reviews: Zoeller 2005
TR binding and transactivation (TRα, TRβ)		Existing: ToxCast/Tox21; Freitas et al. 2011, 2014	Not yet characterized	

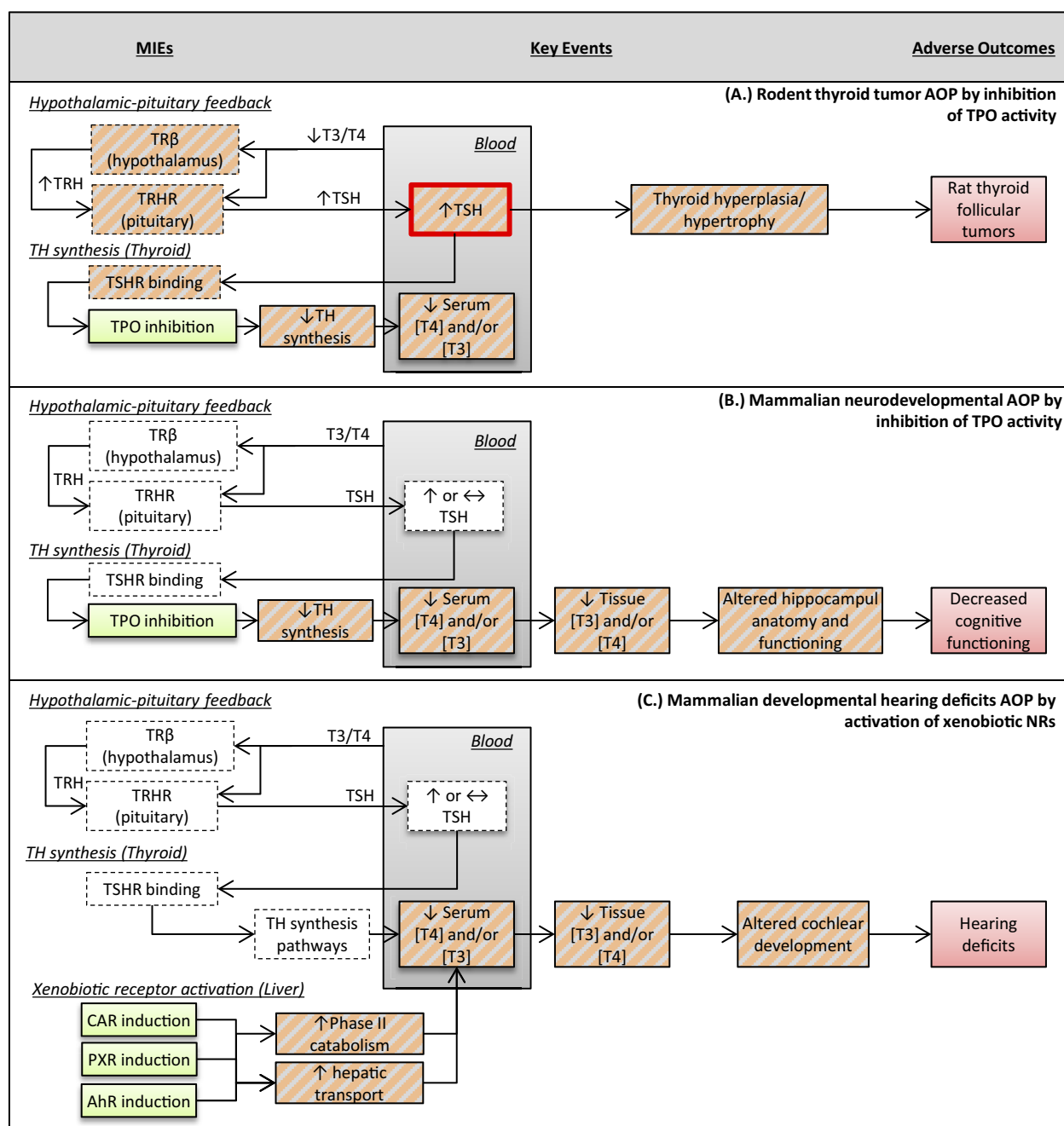


Figure 3. Adverse outcome pathways (AOPs) for select chemical pathways of thyroid disruption, including (A) rat thyroid follicular cell tumors linked to chemical inhibition of TPO; (B) impaired cognitive functioning in mammals linked to chemical inhibition of TPO; and (C) hearing deficits in mammals linked to activation of xenobiotic NRs. In the left-hand column, MIE boxes activated for a given pathway are shown with solid borders (shaded green) and KEs in the AOPs are shown as boxes with striped background (shaded orange). The causative KE in the formation of rat thyroid tumors (A) is increasing TSH (shown with thick borders and striped background and shaded orange) leading to hypothalamic–pituitary compensatory feedback responses shown in left-hand MIE boxes and downstream thyroid hypertrophy and hyperplasia with striped background (and also shaded orange). In contrast, elevated TSH (shown with a dashed border) has not been shown as a causative KE in chemical inhibition of TPO leading to cognitive impairments in mammals (B) or chemical activation of the hepatic xenobiotic NR response cascade leading to mammalian hearing deficits (C). References supporting these AOPs can be found in Table 2. ↔, no change from reference controls; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; KE, key event; MIE, molecular initiating event; NR, nuclear receptor; PXR, pregnane X receptor; T3, 3,3',5-triiodothyronine; T4, thyroxine; TPO, thyroperoxidase; TH, thyroid hormone; TR, thyroid hormone receptor; TRH, thyrotropin releasing hormone; TRHR, thyrotropin releasing hormone receptor; TSH, thyroid stimulating hormone; TSHR, thyroid stimulating hormone receptor; UDPGT, uridine diphosphate glucuronosyltransferase.

anchors representing adverse outcomes (Buesen et al. 2017; Bourdon-Lacombe et al. 2015; Brazma et al. 2001; Dean et al. 2017; Thomas et al. 2013a, 2013b; Villeneuve et al. 2012; Wang et al. 2016).

Assembling and interpreting the scientific evidence measured in independent *in vitro*, *in silico*, and *in vivo* research efforts presents an enormous challenge for chemical assessment. The

thyroid AOP network provides architecture to begin mapping and integrating diverse data to characterize chemical interactions with the thyroid axis. New *in vitro* technologies incorporated into pathway-based approaches are anticipated to help reduce uncertainties and resolve some of the major challenges that are examined below and presented by these evaluations.

Regulatory Challenges in Evaluating and Linking Thyroid MIEs to Adverse Outcomes

Currently, no U.S. EPA or OECD *in vitro* regulatory guidelines test chemical interactions with MIEs in the thyroid axis (Table 1). For most regulatory requirements, hazards are identified for individual chemicals and there are little to no data describing the combined effects of individual chemicals and chemical mixtures that interact with multiple MIEs in the thyroid axis. In addition, current guidelines can detect some thyroid-related disturbances (e.g., changes in serum TH) that have been used to derive hazard values for regulatory purposes, but it is not possible to attribute an adverse outcome to a mechanism. Neither have intermediate parameters (KE and KE relationships) linking an adverse outcome to an upstream MIE been empirically quantified in most cases.

By depicting points where several MIEs converge at shared KEs, the AOP network helps to identify nodes around which assays can be developed to link MIEs to adverse outcomes and clarify data that would be most relevant for regulatory applications. For example, uncertainties continue as to dose–response relationships in chemical-induced changes in serum TH, particularly milder TH perturbations that result in adverse outcomes (Crofton 2004). For example, AOPs (Figure 2; Table 2) describe chemical inhibition of NIS and TPO leading to reduced TH production and decreased serum T4 that culminate in delayed/arrested amphibian development (AOP 176, AOP 175, respectively) and impaired mammalian neurodevelopment (AOP 134, AOP 42, respectively). However, a quantitative understanding of KE relationships in the TPO- and NIS-related AOPs is limiting, although current efforts are beginning to quantify these interactions (Gilbert and Sui 2008; Hassan et al. 2017; O'Shaughnessy et al. 2018b). Decision makers can use the biological plausibility embedded in the thyroid AOP network to clarify linkages for characterization and identify gaps in available knowledge. From this information, KE relationships can be proposed for quantification to support predictive modeling and hazard assessment, as well as reduce data uncertainties.

Differential Species and Life Stage Sensitivities; Interpreting Biomarkers

Future work to expand and refine the thyroid AOP network can help to identify physiological similarities and differences between KEs and KE relationships spanning vertebrate classes. The characterization of shared toxicity mechanisms leading to divergent adverse outcomes across species can be useful in the translation of research between nonmammalian and mammalian species including humans. For example, chemical inhibition of thyroid gland TPO can reduce serum and tissue TH that alter tissue-specific genomic signaling and elicit species- and life stage-specific adverse outcomes [e.g., abnormal brain development in mammals (Zoeller and Crofton 2005); impaired metamorphosis in amphibians (Coady et al. 2010; Hornung et al. 2015; Tietge et al. 2010); reduced anterior swim bladder inflation in teleost fish (Nelson et al. 2016; Stinckens et al. 2016)]. Identification of shared KEs as integrative nodes facilitates characterization of diagnostic biomarkers across species that can be represented in predictive modeling and evaluated in organismal assays to further support mechanistic linkages between KEs and adverse outcomes (Crofton 2008; Miller et al. 2009; Paul et al. 2013; Perkins et al. 2013).

Several MIEs in the thyroid AOP network may induce a cascade of KEs proceeding through decreased serum T4 and/or triiodothyronine (T3) (Figure 2; box with striped background and shaded yellow). Changes in serum TH are a useful biomarker of

thyroid perturbation and are usually correlated to changes in tissue TH (as reviewed by Oppenheimer and Schwartz 1997; Porterfield and Hendrich 1993). However, reliance on serum TH may oversimplify subsequent linkages to downstream KEs that drive adverse outcomes. Serum TH levels are not always aligned with tissue levels as tissue TH is regulated by DIO enzymes, cellular transporters, and regional TR expression. The disconnect between altered TH signaling in the fetal brain, neurodevelopmental consequences, and serum TH has been demonstrated using several gene knock-out (KO) mouse models with deficiencies in genes encoding DIO enzymes, TRs, monocarboxylate transporter (MCT)8, organic anion transporter polypeptide (OATP)1c1, and other TH transporters (as reviewed by Báñez-López et al. 2018; Bernal 2018; Hernandez et al. 2010; Richard and Flamant 2018). For example, considerable evidence describes the contribution of DIO enzymes, particularly DIO2 and DIO3, in the maintenance of T3 in TH-responsive regions of the developing brain [i.e., DIO2 predominantly in astrocytes; DIO3 predominantly in neurons; as reviewed by Bianco et al. (2002), Guadaño-Ferraz et al. (1997), Morreale de Escobar et al. (2008), Patel et al. (2011), and Zoeller (2010)]. However, studies show that *Dio2* and *Dio1/Dio2* KO mouse models with deficient tissue capacity to produce T3 do not experience substantial deficits in motor, learning, or memory functioning, although other deficits (e.g., impaired cochlear and visual development) do occur (Galton et al. 2007, 2009, 2014; Obregón et al. 1991; Schneider et al. 2001; Schneider et al. 2006). These *Dio1/Dio2* KO strains exhibit regionally specific reductions in brain T3 but normal serum T3, as well as elevated serum and tissue T4. In contrast, the *Dio3* KO fetus and newborn pup exhibit an initial hyperthyroidism followed by hypothyroidism, impaired growth and fertility, and reduced survival. Elevations in serum and brain T3 are accompanied by an initial acceleration and subsequent delay of T3-inducible gene expression in the brain (Hernandez et al. 2010). Subsequent experiments have shown that *Dio3* KO mice with augmented brain TH exhibit hyperactivity and reduced anxiety-like behaviors despite systemic hypothyroidism as indicated by reduced serum T3 (Stohn et al. 2016).

As these examples illustrate, the lack of change in serum TH is not a guarantee that TH effects in tissues are not occurring, and conversely changes in serum TH are not always predictive of adverse outcomes. Possible explanations for the lack of concordance between serum TH and adverse outcomes may relate to the not yet fully characterized age- and regional-specific responses of the brain to compensate to systemic TH insufficiency. Nonetheless, fetal and early postnatal susceptibilities to thyroid toxicants are heightened because their thyroid feedback systems are absent or incompletely formed and they have low TH reserves that are critical to neurological development (as reviewed by Morreale de Escobar et al. 2004a; Skeaff 2011; Williams 2008; and Zoeller and Rovet 2004). The thyroid AOP network provides a tool to map linkages and identify possible KEs for further characterization and quantification (e.g., trapezoid boxes also shaded blue in Figure 2) to aid in predicting adversity. For purposes of decision making, changes in serum TH profiles are not proscriptive to either mechanisms or outcomes, but they nevertheless do provide clear indication of perturbed thyroid homeostasis with the potential to adversely affect development.

The thyroid AOP network also can identify KEs that may not fall on the causative pathway leading to an adverse outcome, making these KEs potentially less relevant to decision making. One such example is the stimulation of the thyroid–pituitary feedback response (i.e., increased TSH). Although TSH increases often accompany exposure to a number of thyroid disrupting chemicals, TSH is not a causative KE in AOPs involving TPO inhibition that result in impaired hippocampal development and cognitive deficits (Zoeller and Crofton 2005) and up-regulated

TH catabolism leading to ototoxicity (Crofton and Zoeller 2005) (Figure 3). For example, rat offspring developmentally exposed to some polychlorinated biphenyls (PCBs) and dioxins experience mainly hypothyroxinemia (i.e., reduced serum T4 with TSH in reference control ranges) that culminate in impaired cochlear development and hearing loss (Crofton et al. 2000; Crofton 2004; Goldey et al. 1995; Morse et al. 1993; Sher et al. 1998). Elevated TSH in rodents leads to thyroid hypertrophy and potential thyroid cancer, an adverse outcome that has limited relevance to human thyroid cancer due to species differences in sensitivity (Capen and Martin 1989; EU 2017; Hurley 1998; McClain et al. 1988), although this too is an area of renewed interest (EU 2017). Thus, elevated TSH may not be a reliable KE biomarker for thyroid cancer in humans, but increased TSH in rodents does indicate a chemical capable of perturbing TH signaling in other species, including humans, and is involved in other thyroid-responsive outcomes, including neurodevelopment.

Understanding pathway conservation is critical for cross-species extrapolation and requires understanding the conservation of protein targets (e.g., MIEs) and resulting sensitivity to chemicals. Research efforts in this area range from *in vivo* exposures across phylogenetically disparate species, followed by toxicogenomic meta-analyses (Garcia-Reyero et al. 2011), to bioinformatics comparing amino acid sequences/functional domain similarity across species to identify putative functional orthologues (LaLone et al. 2013). For purposes of chemical screening and *in vitro* HTS assay development, a basic demonstration of cross-species concordance of chemical activity is sufficient to provide confidence that the representative *in vitro* system is reliable for generating screening-level data. For example, strong cross-species concordance in the activity of porcine and rat TPO has been shown despite only 75% amino acid similarity in the conserved peroxidase domain (Paul et al. 2013). Results from numerous *in vivo* exposures to select TPO-active chemicals in fish (Doerge et al. 1998; Nelson et al. 2016; Stinckens et al. 2016), amphibians (Coady et al. 2010; Hornung et al. 2015; Tietge et al. 2010, 2013), birds (Grommen et al. 2011; Rosebrough et al. 2006), and mammals (Davidson et al. 1978; Divi and Doerge 1996; Francis and Rennert 1980) corroborate the conserved mechanistic linkages leading to species-specific adverse outcomes. Such information can define circumstances under which screens for thyroid activity can benefit from combining lines of evidence across species with shared KEs, as opposed to situations when species-specific data support being separated for regulatory use.

Human Relevance of Hepatic- and Serum Distributor-Driven TH Effects in Rodents

The observation of thyroid-related end point changes in rodents and their relevance to predicting adverse outcomes in humans continues to present research questions. One such data gap concerns interpreting the human applicability of chemical-induced TH reductions in rodents through activation of xenobiotic NRs, notably PXR and CAR, and up-regulation of hepatic TH catabolism (Figures 2–3; Table 2) (EU 2017; ECHA 2016). Although functionally conserved in many cases (e.g., CYP450s), hepatic drug metabolizing enzymes have differing isoform compositions, substrate selectivity, and catalytic activities depending on species, life stage, and genetics that may affect chemical and TH biotransformation (Benedetti et al. 2005; Curran and DeGroot 1991; Kondo et al. 2017; Martignoni et al. 2006; Nebert and Gonzalez 1987).

Increased hepatic catabolism of TH, along with reductions in serum TH and elevated TSH, are well described in rodents (rat, mouse, hamster) exposed to hepatic enzyme-inducing chemicals such as phenobarbital, some PCBs (Aroclor 1254), pregnenolone-16 α -carbonitrile, and 3-methylcholanthrene (Barter and Klaassen

1994; Haines et al. 2018; Hood et al. 2003; Kato et al. 2010; Klaassen and Hood 2001; Vansell and Klaassen 2002; Wong et al. 2005). Studies in human subjects also demonstrate the responsiveness of TH catabolic pathways to hepatic enzyme inducers (Benedetti et al. 2005; Christensen et al. 1989; Curran and DeGroot 1991; Ohnhaus et al. 1981; Rootwelt et al. 1978; Yeo et al. 1978; Zhang et al. 2016). In contrast with rodents, human serum T4 decrements reported in these studies are varied and typically not accompanied by changes in TSH, suggesting the possibility of milder responses in humans compared with rodents, although this may not be the case in sensitive subpopulations. For example, studies in healthy human test subjects receiving therapeutic doses of the human CAR activator phenobarbital and other antiepileptic drugs show unchanged or decreased serum TH, with generally no TSH alterations (as reviewed by Benedetti et al. 2005; Curran and DeGroot 1991). Similarly, healthy human subjects receiving therapeutic doses of the antibiotic and human PXR agonist rifampicin exhibit reduced serum T4, as well as increased T4 and rT3 clearance, with no change in TSH (Christensen et al. 1989; Ohnhaus et al. 1981). However, a meta-analysis of published clinical studies that examined TH end points in epileptic patients treated with phenobarbital and other antiepileptic drugs detected an overall decrease in serum T4 (no change in T3) and increase in TSH (Zhang et al. 2016), suggesting genetics and health status may influence sensitivity. The antibacterial triclosan, known to decrease serum TH in rats, has also been shown to activate human PXR, but not rat or mouse PXR, and to behave as an inverse agonist to human CAR1 and weak agonist of human CAR3 and rat/mouse CAR (Paul et al. 2013). Thus, current evidence supports that chemicals may induce hepatic TH catabolism in both humans and rodents, albeit by possibly differing metabolic pathways and potencies (an unknown at this time). Whether responses in humans extend to other chemicals as a general phenomenon is unclear, and differing exposure durations and responses between rodents and humans may be relevant to extrapolations from one species to another. Useful follow-up analyses of chemicals suspected of NR activation could involve evaluation of species selectivity of the NR response to the chemical itself.

It has also been posited that TH metabolic clearance responses in rats are less relevant to humans because TBG, the major serum TH distributor protein in humans, is less prominent in the adult rat, although it does play an important role in thyroid homeostasis in newborn and neonatal rat pups (Savu et al. 1991; Young et al. 1988). In adult rodents, amphibians, birds, and fish, TTR appears to be the major serum TH distributor protein (Dickson et al. 1985; Harms et al. 1991; Power et al. 2000). TTR has a lower binding affinity for T4 than TBG, and this is thought to be responsible for the shorter T4 half-life in rats (~12–24 h) than in humans (~5 d) (Lewandowski et al. 2004). Unlike TBG, TTR is important in the transport of T4 across the blood–placental (Landers et al. 2009; Mortimer et al. 2012), blood–brain (Chanoine et al. 1992; Schreiber et al. 1990), and CSF–brain (Richardson et al. 2018) barriers. Thus, a role for human TTR in TH storage and transport appears critical during fetal development. Some researchers have also suggested that although TBG binds most TH in human circulation, TH dissociation rates from TTR and capillary transit times make TTR a significant distributor protein of TH in humans as well as in rodents (Alshehri et al. 2015; Mendel 1989). Taken together, current evidence supports the activation of putative MIEs and KEs in rats, encompassing xenobiotic NR activation, inductions of UDPGTs, SULTs, and TH distributor proteins are relevant pathways for consideration. In this regard, the rat serves as a conservative model for predicting human health effects. The thyroid AOP network allows for the integration of new *in vitro* mechanistic and interspecies scaling information to help relate rodent toxicity data

to human-equivalent exposures and further populate the TH catabolic pathway for use in human health assessment.

Building Predictive Models and Toxicokinetics

Because of the diversity of known and putative molecular targets in the thyroid axis, using AOPs to develop predictive models of adverse outcomes from *in vitro* data will be challenging and require additional bioassay data for KEs in the network, particularly downstream of serum TH. Although there is qualitative evidence linking the MIEs listed in Table 2 to altered serum TH, data to establish quantitative KE relationships helpful for regulatory application are lacking. Furthermore, the large diversity of genes regulated directly and indirectly by T3, the nongenomic effects of T4, and our limited knowledge of the functioning of TH during fetal development present a challenge for predicting chemical-induced adverse outcomes from *in vitro* data (as reviewed by Bernal 2018; Richard and Flamant 2018).

In humans, detrimental effects of mild TH insufficiency on fetal cognitive development are recognized as an important public health concern, and some links to chemical exposures are described (Finken et al. 2013; Ghassabian et al. 2014; Haddow et al. 1999; Korevaar et al. 2016; Moleti et al. 2011; Morreale de Escobar et al. 2000; Steinmaus et al. 2016; Vermiglio et al. 2004). The observations in humans are supported by rodent studies where neurodevelopmental impairments result from moderate and transient reductions in serum TH induced by chemical exposures (Ausó et al. 2004; Boyes et al. 2018; Gilbert 2011; Gilbert et al. 2013, 2016, 2017; Morreale de Escobar et al. 2004b). As quantitative data become available for these types of sensitive developmental responses—likely from the use of bioassays of greater biological complexity that integrate multiple MIEs and KEs—additional modeling approaches to further characterize KE relationships could be implemented to predict thyroid-related adverse outcomes and characterize accompanying uncertainties. As a first step, models that integrate *in vitro* assay results for a single KE (when multiple assays exist) and models that integrate *in vitro* assay results for more than one KE in a target tissue (e.g., thyroid gland; TPO, NIS, DIO) could be used to prioritize chemicals for further assessment and targeted *in vivo* testing. Development of a computational systems biology model of thyroid function could help simulate the potential interactions of chemicals with activity at various MIEs. Such an effort will require an investment of research to parameterize and evaluate uncertainties.

Another challenge is that chemical toxicokinetic considerations, and an understanding of potential differences among life stages and species, are not well accounted for by current *in vitro* test systems. These limitations can lead to both false positive and false negative calls in screening efforts. Developing *in vitro* predictions of thyroid perturbations is further complicated by the fact that most chemically induced effects on downstream KEs and adverse outcomes are indirect, secondary consequences to a chemically induced change in hormone regulation. We think predictions of *in vivo* adverse outcomes from *in vitro* HTS data and other new technologies will be greatly facilitated by *a*) incorporation of toxicokinetic tools to estimate metabolic bioactivation and better characterize potency and selectivity of chemicals for target tissues, and *b*) improved mechanistic and quantitative understanding of TH action on development of target organs, especially the brain.

Conclusions

Advancements in new *in vitro* technologies offer opportunities to screen chemicals for interactions with MIEs in the thyroid pathway and to anchor adverse outcomes to a thyroid mechanism. A

substantial body of knowledge exists concerning the molecular and physiological regulation of TH signaling and responses of these systems to chemical exposures (including pharmaceuticals). The thyroid AOP network described in the present work is based on decades of research and multiple consortia to evaluate relevant KEs for chemical-induced thyroid disruption. At present, the use of *in vitro* data beyond screening and prioritization for thyroid bioactivity is challenged by the complexity of potential TH-related adverse outcomes and the limited knowledge of mechanistic processes controlling such responses. Despite these knowledge gaps, we propose that the thyroid AOP network provides the necessary biological structure to organize diverse data from differing test methods, thereby serving as a useful data integration tool to assist in hazard screening for large numbers of previously untested chemicals. As more data become available, the AOP network can be further populated to help bridge knowledge gaps and identify key nodes for assay development.

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