Overview of *In Vitro* Assays to Investigate Chemicals for Thyroid-Axis Disrupting Potential

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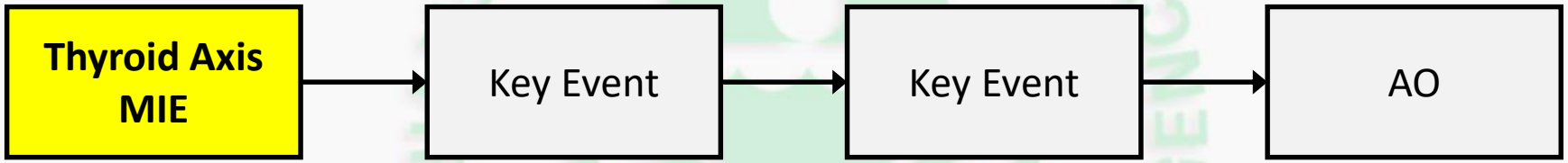
HESI DART Workshop

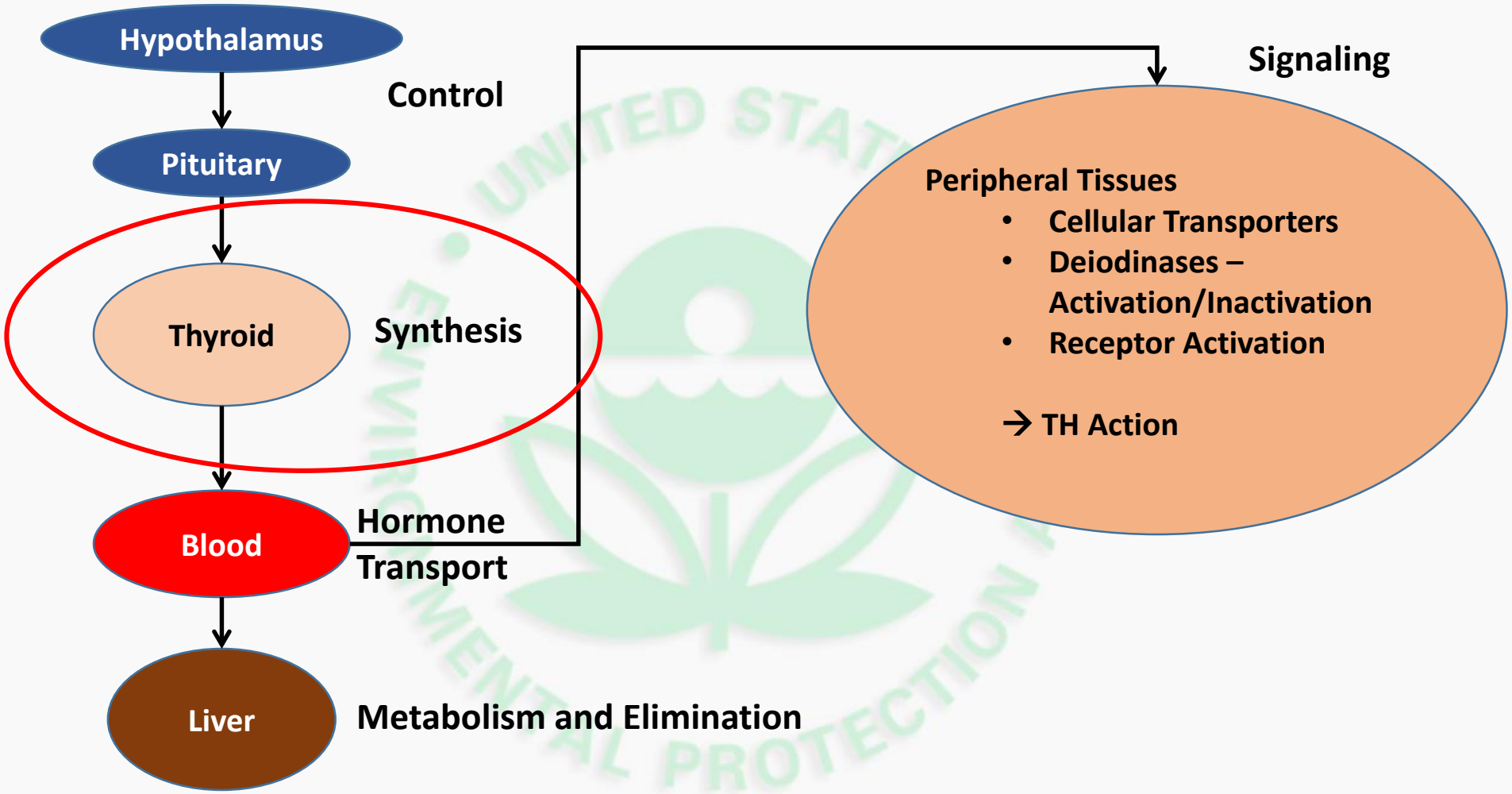
The Problem

- Need to develop screening assays to address Molecular Initiating Events that are most likely to be affected by chemicals to affect thyroid axis function and initiate an AOP



- The aim of this presentation is to provide information on the *in vitro* screening assays available or in development to address the need to identify potential thyroid hormone disrupting chemicals

Thyroid Axis



Thyroid Hormone Synthesis

Synthesis MIEs

<u>Iodide Transport</u>

Sodium-Iodide Symporter (NIS)

Pendrin (PEN)

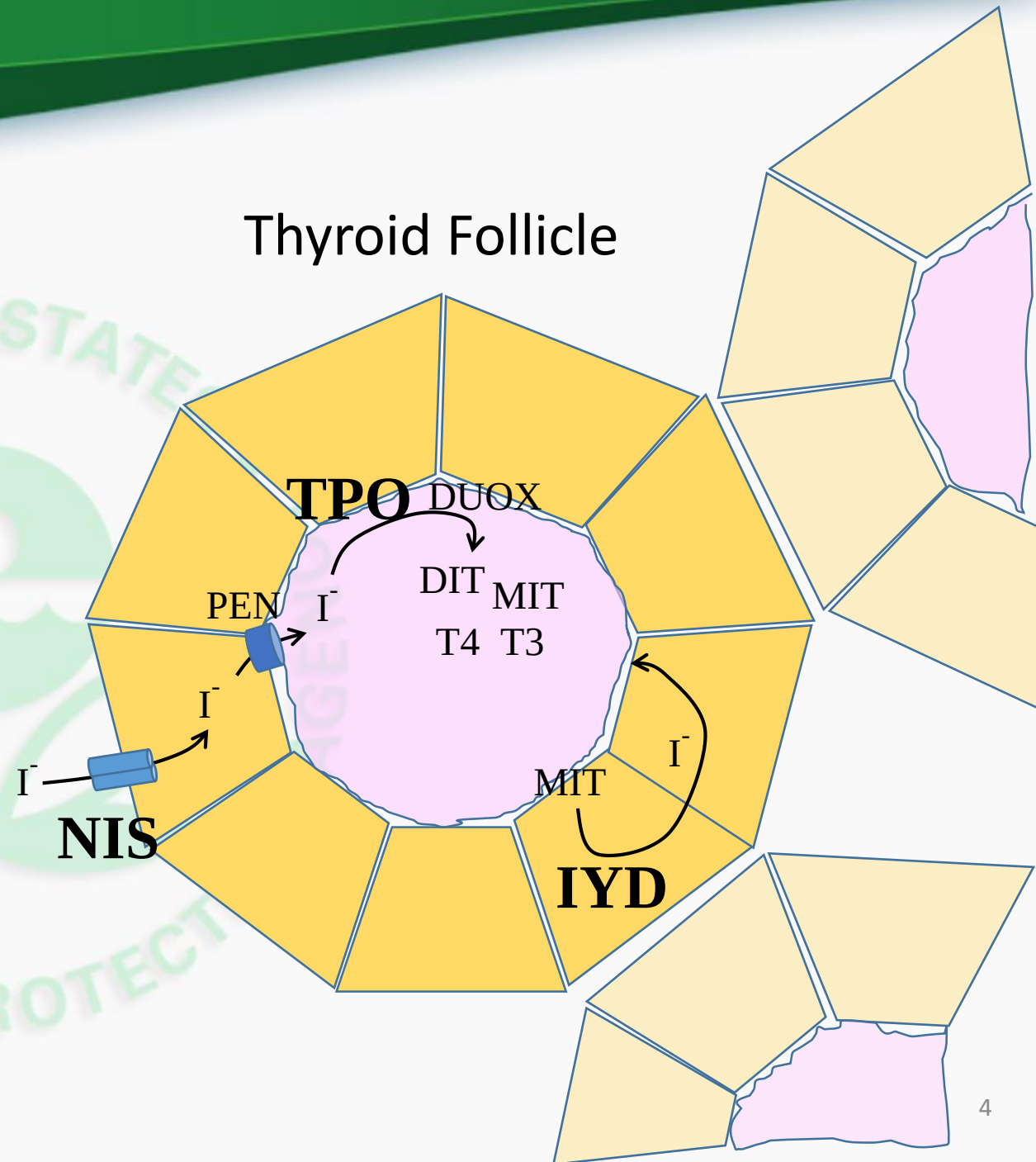
<u>Tyrosyl Iodination and Coupling</u>

Thyroperoxidase (TPO)

Dual Oxidase (DUOX)

<u>Iodide Recycling</u>

Iodotyrosine Deiodinase (IYD)



Thyroid Hormone Synthesis

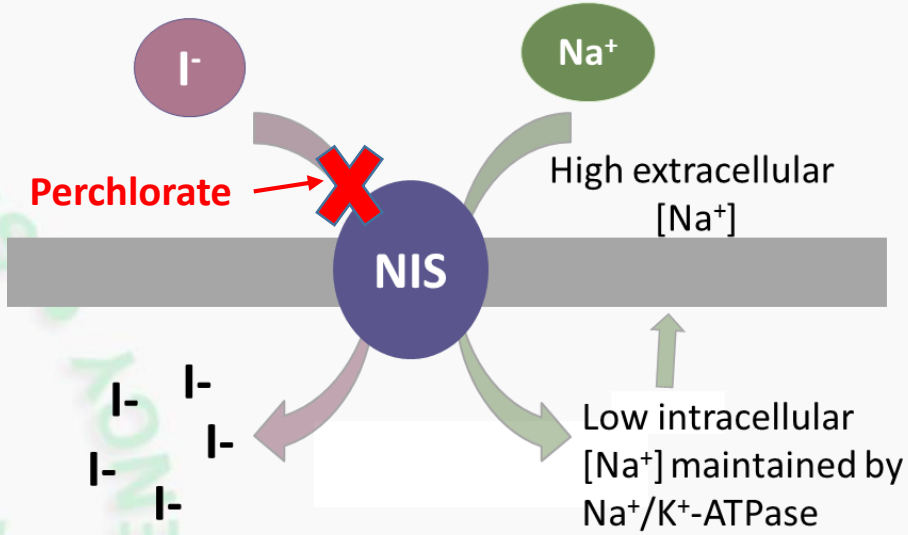
MIE target	Effect	In vitro assay	References
Sodium-iodide symporter (NIS)	Inhibition reduces iodide uptake by thyroid reducing hormone production	**Radioactive iodide uptake inhibition assay (cell-based assay)**	Hallinger et al. 2017 Wang et al. 2018
Thyroperoxidase (TPO)	Iodination of tyrosyls on thyroglobulin and coupling to produce T3 and T4. Inhibition leads to reduced hormone production	**Fluorescent peroxidase substrate with stable signal (cell-free assay)**	Paul-Friedman et al. 2016
Iodotyrosine deiodinase (IYD)	Recycles iodine from MIT and DIT in thyrocytes. Inhibition leads to iodide insufficiency.	**Measurement of iodide release from hormone substrate. Cell-free screening assay in development.**	Shimizu et al. 2013
Pendrin	Transport iodide from thyrocyte to lumen for use by TPO	None developed	
Dual oxidase (DUOX)	Produces peroxide necessary for TPO action.	None developed	

Thyroid Hormone Synthesis

Iodide Uptake Inhibition

NIS = Sodium/iodide (Na^+/I^-) symporter

- Mediates thyroid gland iodide uptake
- Known target of environmental contaminants (ex. Perchlorate, ClO_4^-)
- Limited knowledge for more structurally diverse chemicals



Radioactive Iodide Uptake Assay

Cell Culture hNIS-HEK293T-EPA → Radioactive ^{125}Iodide Uptake X Cell Viability → Measure ^{125}I uptake X Cell Viability by ATP

Thyroid Hormone Synthesis

Iodide Uptake Inhibition

Single-Concentration Screening

1087 Samples
RAIU assay (100 µM)

↓

Selection

> 20% inhibition

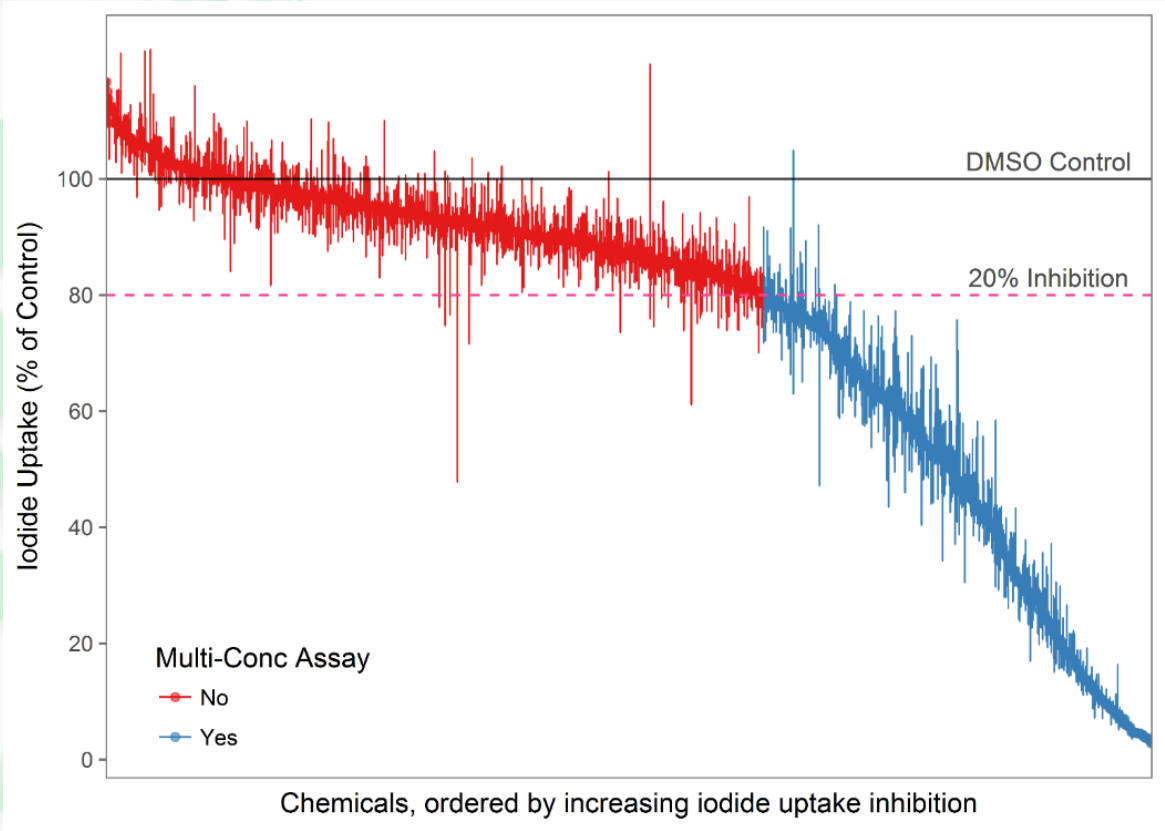
↓

Multi-Concentration Screening

404 Samples
RAIU & Cytotox Assays
(0.001 - 100 µM)

Screening ToxCast Phase I and II Libraries for NIS Inhibition

Median and Range of Test Chemical Responses (Single Concentration Screening)



Wang, J. et al. 2018. Environ. Sci. Technol. 52(9): 5417-5426.

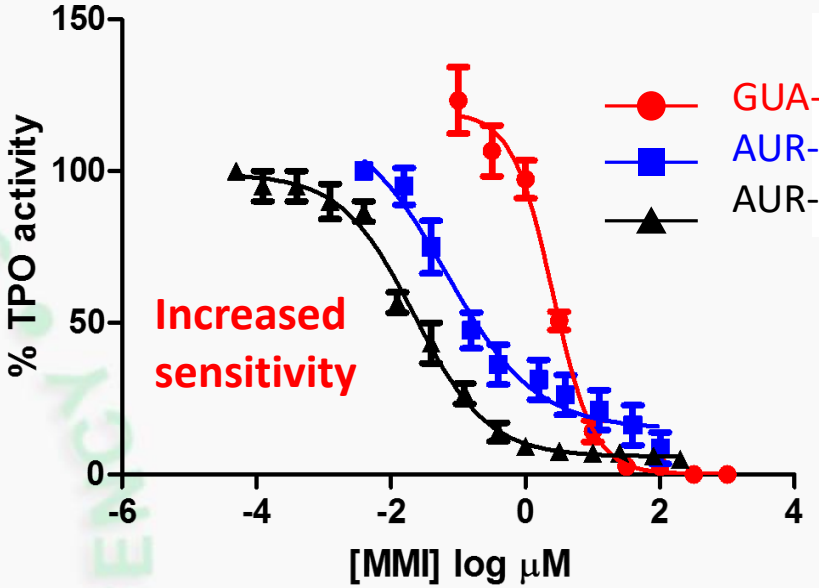
Wang, J. et al. 2019. Environ. Int. 126: 377-386.

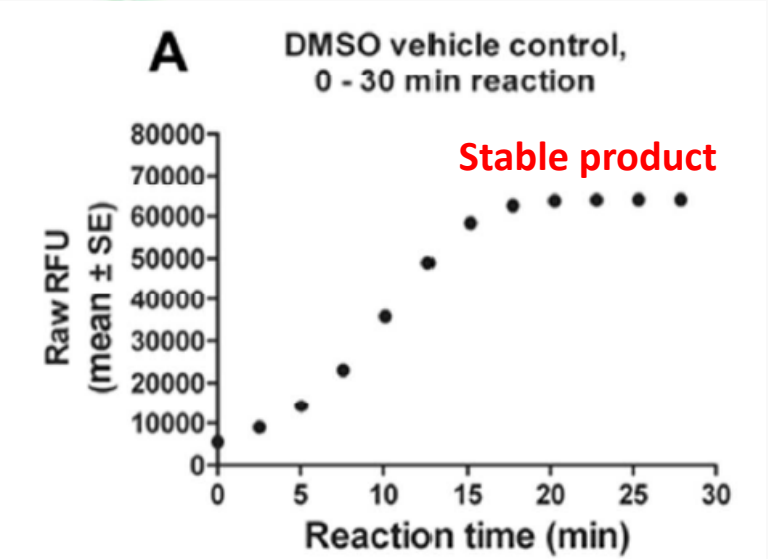
Thyroid Hormone Synthesis

Thyroperoxidase Inhibition

- TPO in the thyroid gland catalyzes tyrosine iodination & coupling in synthesis of T4 & T3.
- Evaluated commercially-available peroxidase substrates to find a substitute for guaiacol that was HTS-amenable:
    - **Amplex UltraRed (AUR)** met criteria needed for a HTS substrate
- Rat thyroid gland microsomes were source of TPO for this screening
- Recombinant TPO source is promising

New AUR vs. Legacy GUA



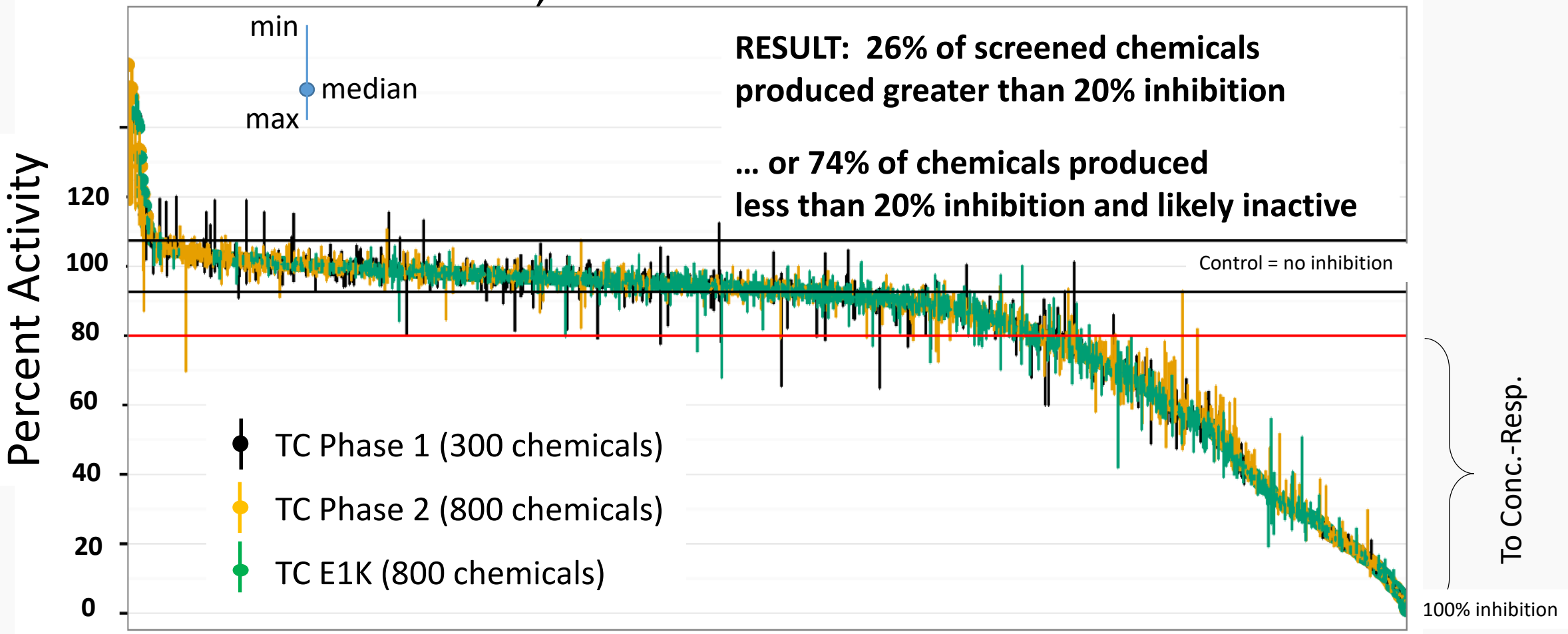


Paul et al. (2014). *Chemical Research in Toxicology* 27(3), 2014, 387-399.

Thyroid Hormone Synthesis

Single-concentration screen for TPO inhibition activity:

1,900 ToxCast chemicals

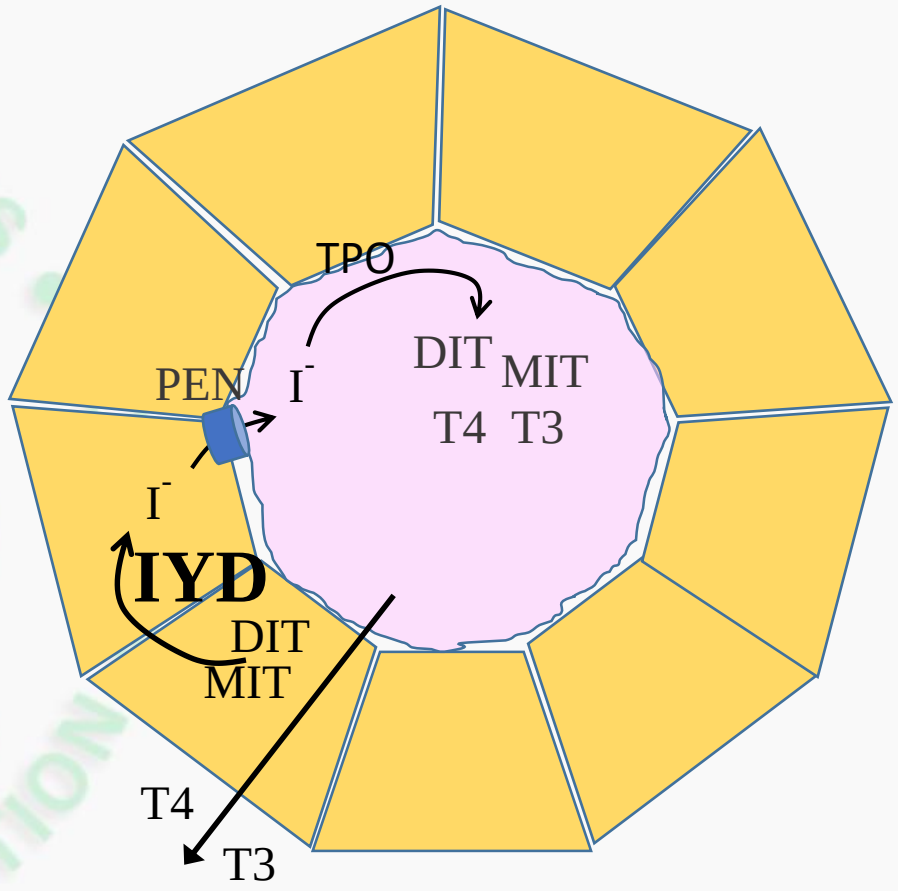


Paul et al. (2016). *Toxicological Sciences* 151(1), 2016, 160–180.

Thyroid Hormone Synthesis

- **Iodotyrosine Deiodinase (IYD) Inhibition**
  - MIT and DIT are deiodinated in follicular cells, thereby recycling iodide for TH synthesis
  - Human IYD deficiency/mutation leads to adverse developmental clinical consequences. (iodotyrosine deiodinase deficiency disorder).
  - IYD identified as important MIE in amphibians
    - Olker et al. 2018. Toxicol. Sci. 166, 318-331.
  - In vitro assays are being developed for human and amphibian IYD.
    - Shimizu et al. 2013. Toxicology 314(1): 22-29.

Thyroid Follicle



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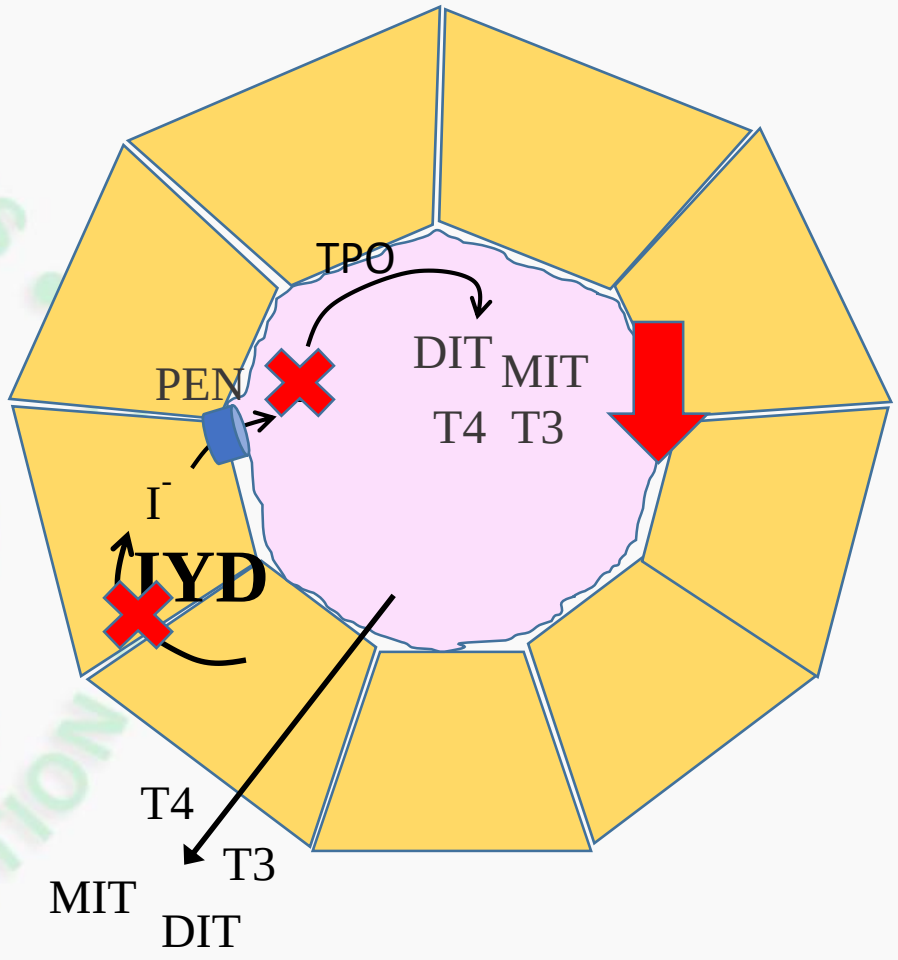
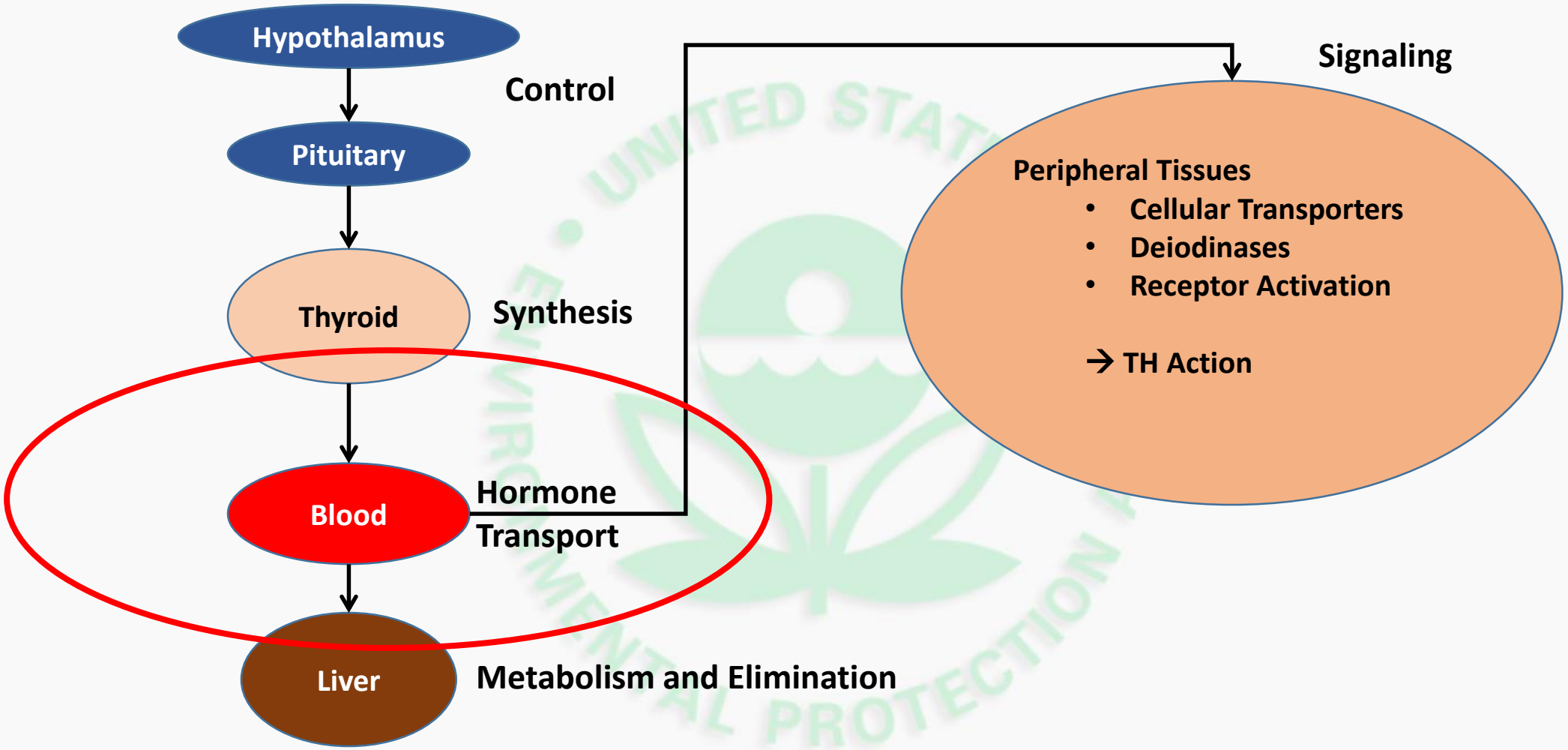


Molecular Initiating Events



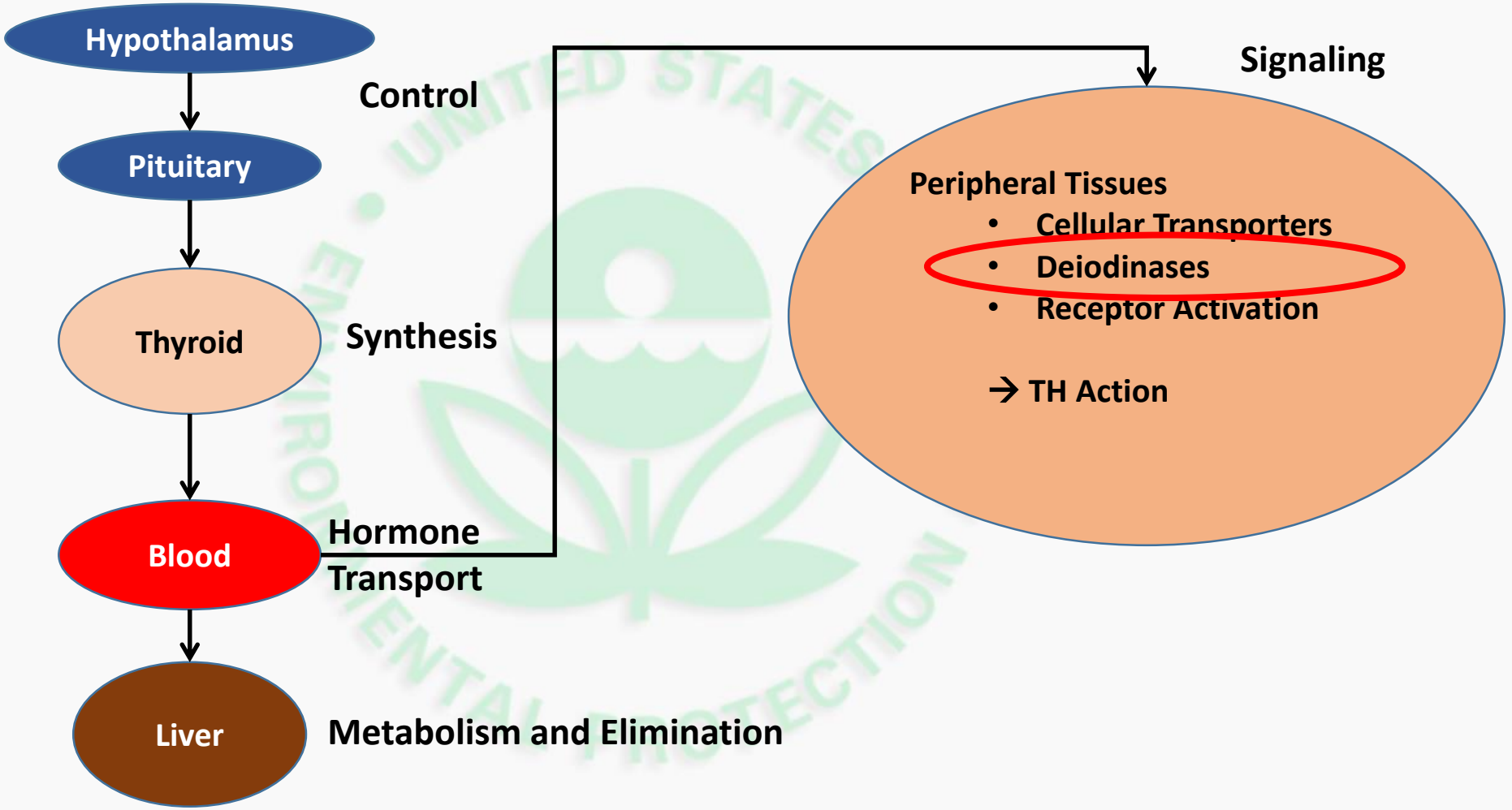
Thyroid Hormone Transport

MIE target	Effect	In vitro assays	References
Thyroid binding globulin Transthyretin Serum albumin	Chemical competition for thyroid hormone binding to serum reduces available hormone to peripheral tissues	"Competitive binding assays" with radioactive (RIA) or non-radioactive iodide	Marchesini et al. 2008 (plasmon resonance)

- Thyroxine Binding Globulin – responsible for binding 75% of T4 in human
- Transthyretin – 20% of T4, but important for blood-placental and blood–brain transport (and primary transporter in rodents, and non-mammalian vertebrates)
- Albumin – 5%, non-specific

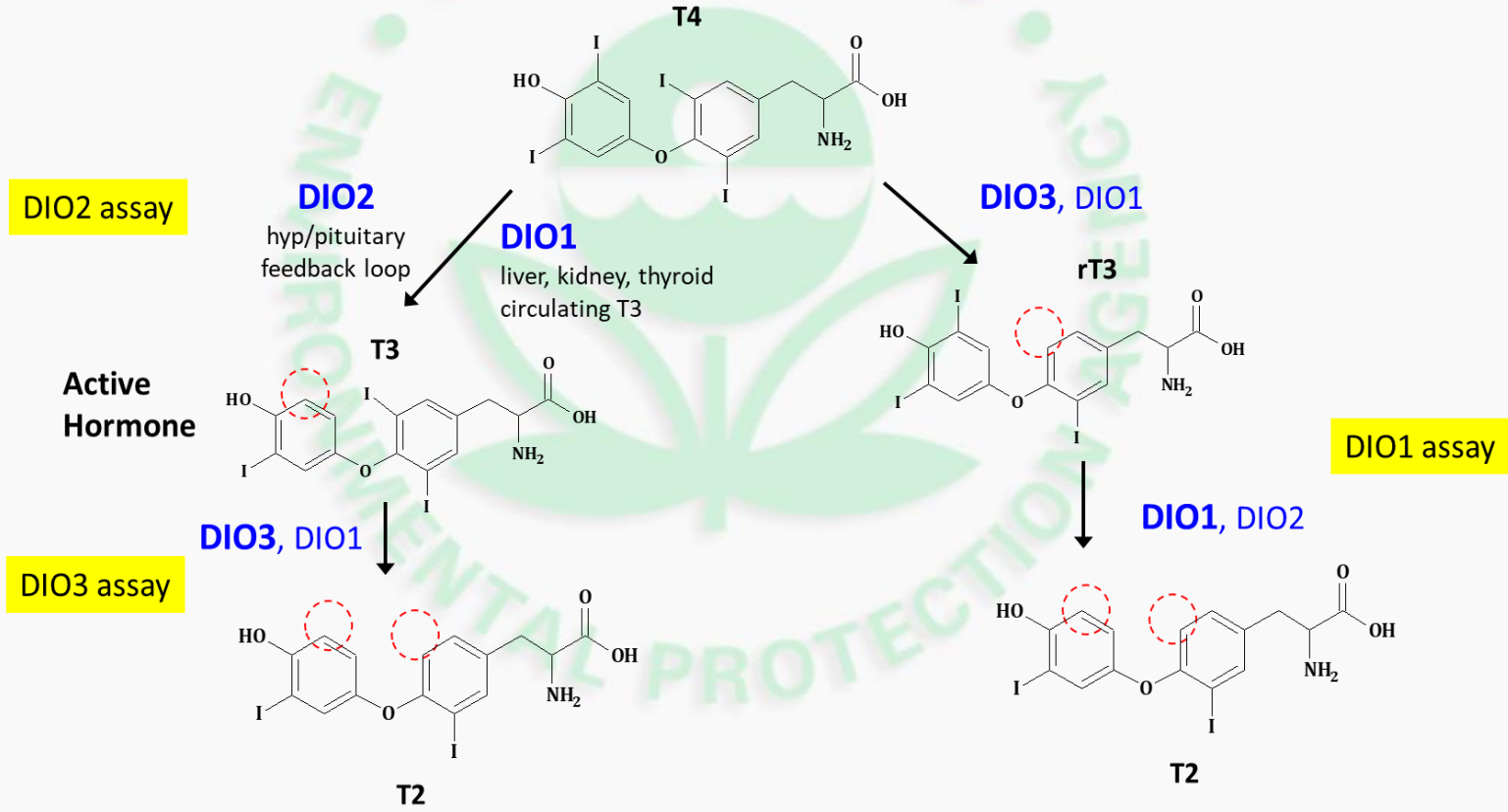
Molecular Initiating Events

- Metabolic Activation / Inactivation



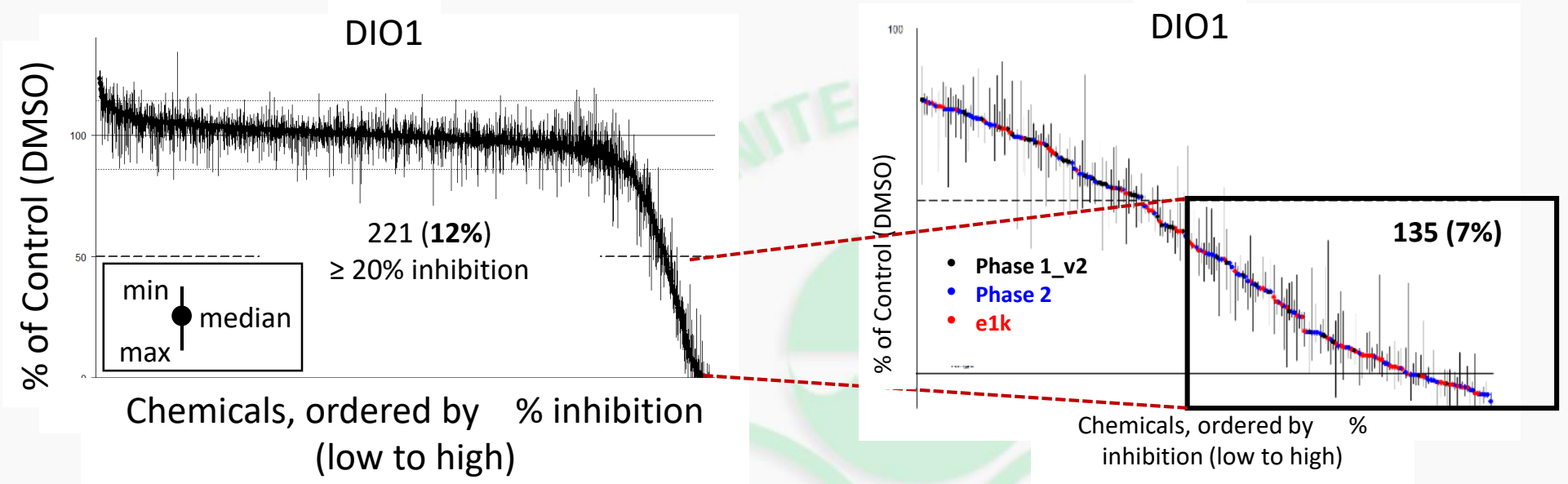
Hormone Activation/Inactivation

MIE target	Effect	In vitro assay	References
Iodothyronine Deiodinases (DIO1, DIO2, DIO3)	Alter normal activation and inactivation of T4 and T3 in tissues.	Iodide release from hormone substrate; recomb. enzymes	Renko et al. 2012. Olker et al. 2019.



Hormone Activation / Inactivation

- Deiodinase Inhibition Assay

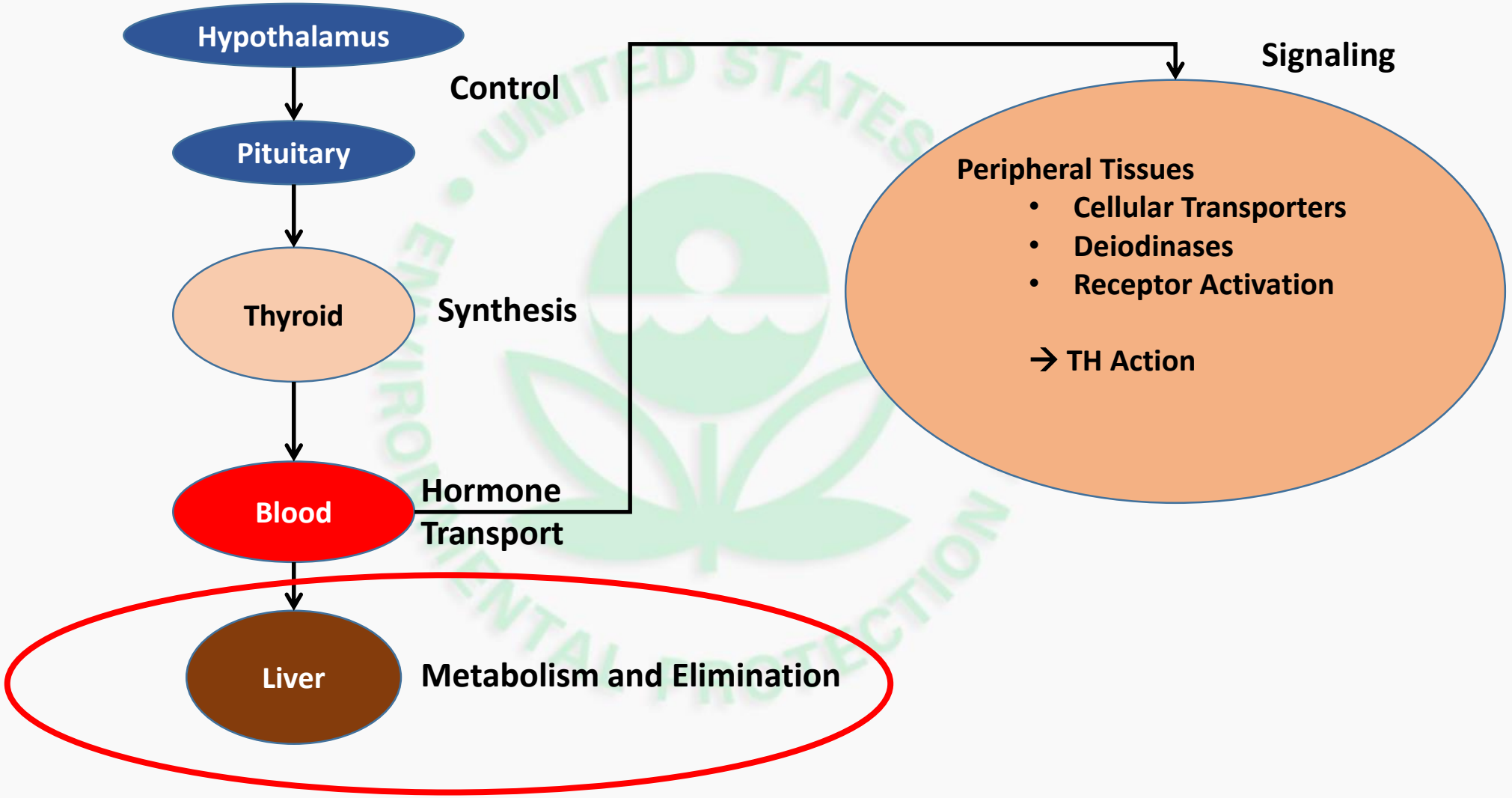


		Deiodinase Type 1		Deiodinase Type 2		Deiodinase Type 3	
Chemical Library	# chemicals tested*	# with ≥ 20% inhibition	% with ≥ 20% inhibition	# with ≥ 20% inhibition	% with ≥ 20% inhibition	# with ≥ 20% inhibition	% with ≥ 20% inhibition
ToxCast p1_v2	290	49	16.9 %	54	18.6 %	57	19.7 %
ToxCast p2	748	95	12.7 %	126	16.8 %	117	15.6 %
ToxCast e1K	781	77	9.9 %	123	15.8 %	133	17.1 %
Total	1,819	**221**	**12.1 %**	**303**	**16.7 %**	**307**	**16.9 %**

Olker et al. 2019. Toxicol. Sci. 168(2); 430-442

Molecular Initiating Events

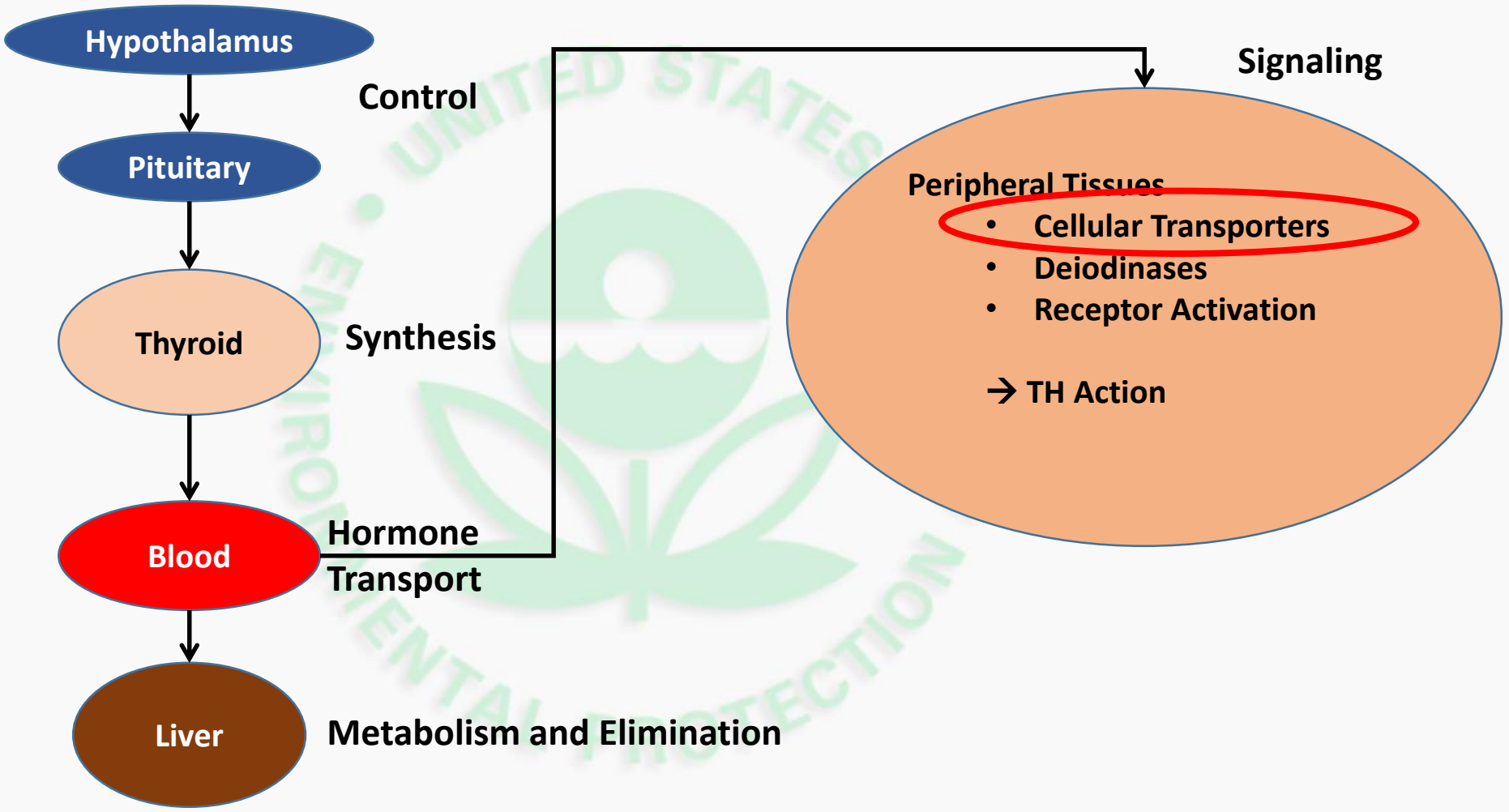
- Metabolism and Elimination



Hormone Metabolism and Elimination

MIE target	Effect	In vitro assay	References
Constitutive androstane receptor (CAR); Pregnane X receptor (PXR); Aryl hydrocarbon receptor (AhR)	Increased catabolism of thyroid hormones	ToxCast/Tox 21 transactivation assays available	He et al. 2011; Maglich et al. 2003; Romanov et al. 2008;
Uridine diphosphate glucuronosyltransferase (UDPGTs; e.g., UGT1A1, UGT1A6); Sulfotransferases (SULTs; e.g., SULT2A1)	Increased catabolism of thyroid hormones	In development	
Peroxisome proliferator-activated receptor (PPARα, PPARβ/δ, PPARγ)	Activation of PPARs potentially compete for RXR as heterodimer with THR's.	ToxCast/Tox 21 transactivation assays available	Huang et al. 2016; Martin et al. 2010; Romanov et al. 2008

Molecular Initiating Events

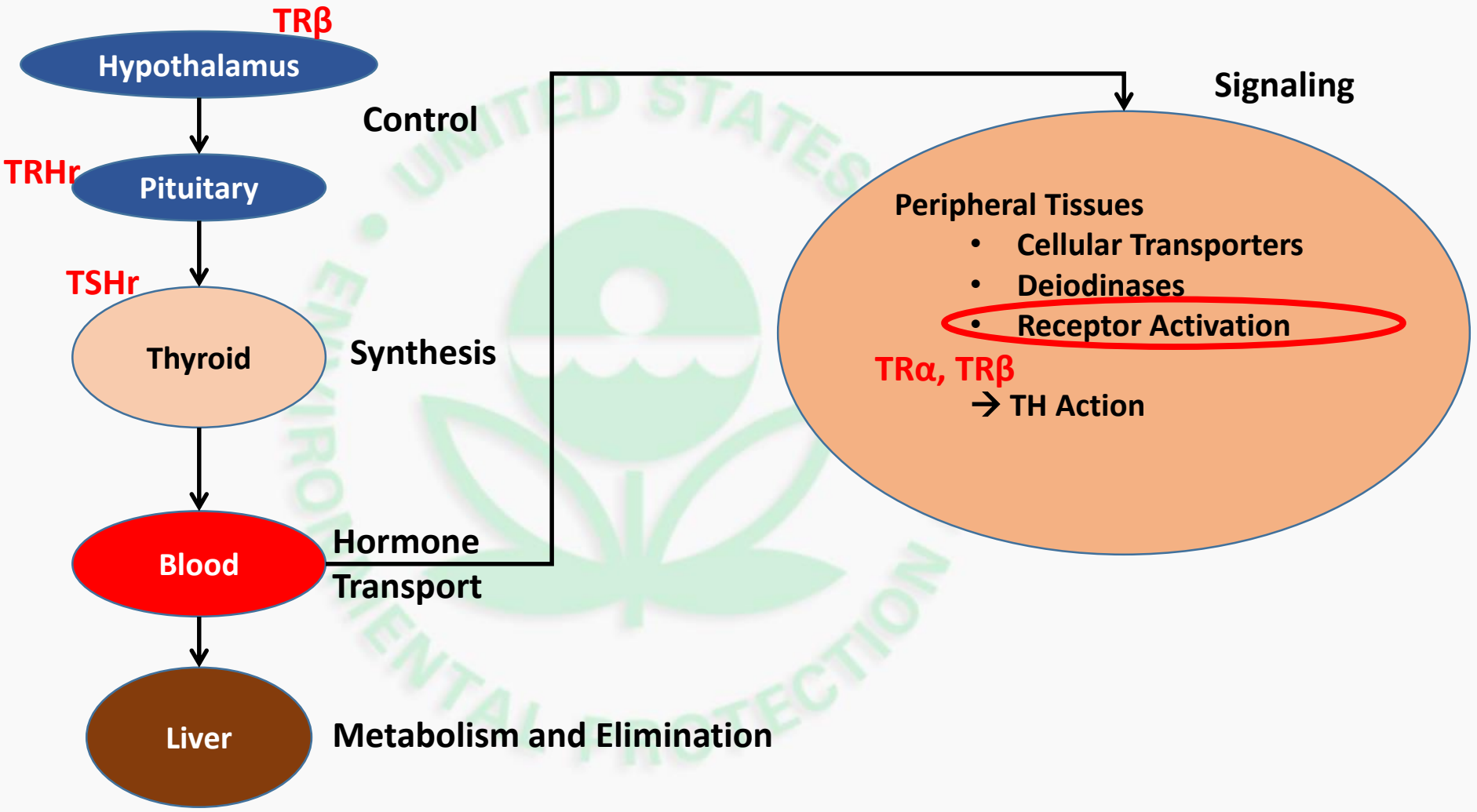


Molecular Initiating Events

- Cellular Transporters

MIE target	Effect	In vitro assay	References
Monocarboxylate transporter (MCT8, MCT10);	Specific transporters for TH. Mutations produce adverse effects. Information on chemical interaction with these is limited.	Potential; endpoint measure hormone (via analytical chem) or iodide (*I or I)	Jayarama-Naidu et al. 2015; Dong and Wade 2017;
Organic anion transporter polypeptide (e.g., OATP1C1; OATP1A4			

Molecular Initiating Events



Molecular Initiating Events

- Receptor binding and transactivation

MIE target	Effect	In vitro assay	References
TRH receptor	Stimulates synthesis and release of TSH	ToxCast/Tox21	
TSH receptor	Activates thyroid hormone synthesis pathways in thyroid gland	ToxCast/Tox21	Paul-Friedman et al. 2017 (SOT poster)
TR binding and transactivation (TRα, TRβ)	Chemical screening to this point indicates chemicals that can affect TH receptor binding and activation are limited. OH-bisphenols, biphenyls, diphenylethers.	ToxCast/Tox21	Freitas et al. 2014; Moriyama et al. 2002; Romanov et al. 2008

Non-mammalian assays

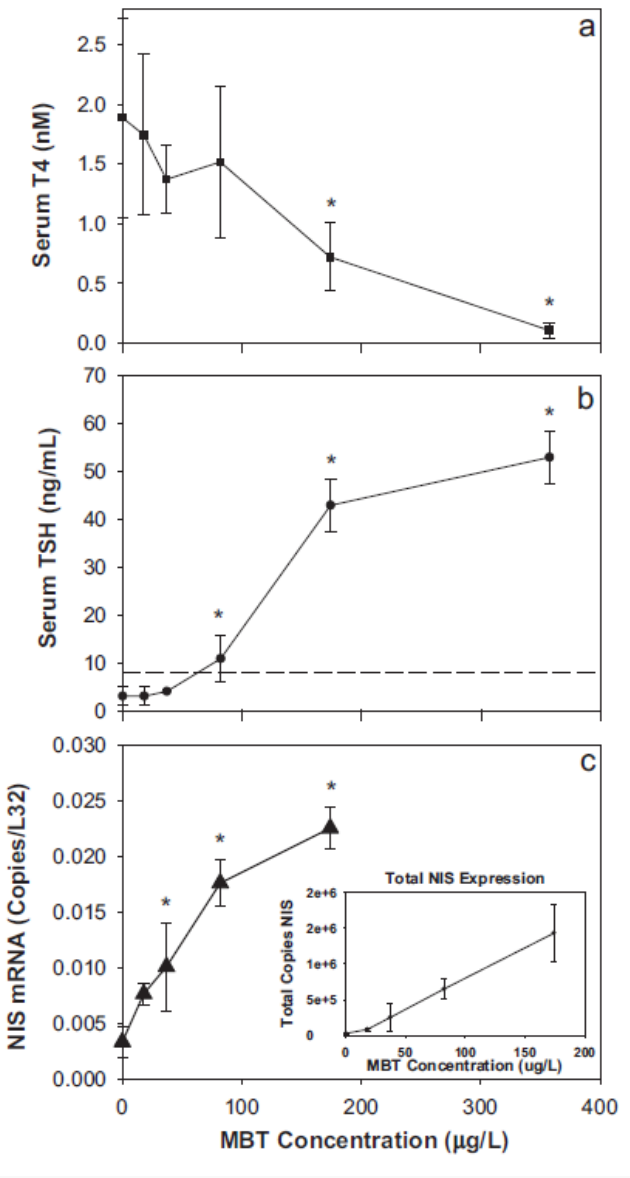
- In Vivo Harmonized Assays
  - Amphibian Metamorphosis Assay
    - EPA 890.1100 / OECD TG 231
  - Larval Amphibian Growth and Development Assay
    - EPA 890.2300 / OECD TG 241

    Developmental progression and impaired metamorphosis are indications of thyroid hormone disruption, with thyroid gland histology as diagnostic endpoint
- Avian two-gen toxicity test: EPA 890.21000
  - TSH, T4, thyroid weight & histology endpoints.
- Non-mammalian higher throughput screening assays are very limited.
  - Xenopus tadpole thyroid receptor screen
  - In development: Xenopus deiodinases

Non-mammalian assays

- **Shorter term (7d) assays with Xenopus laevis have been conducted that include additional diagnostic endpoints (TSH, NIS expression, thyroid gland and blood hormone levels).**
  - Tietge, JE, Degitz, SJ, Haselman, et al. (2013). *Aquat. Toxicol*. 126: 128-136.

- **Sensitive UHPLC-MS/MS methods for thyroid hormone analysis of small tissue (single thyroid gland ~ 1 mg) and blood samples (< 25 µL)**
  - **Limit of detection ~ 0.02pmol/glands; ~ 60 pM for serum TH. 0.02-0.05 ng/ml LOQ in rat serum**
    - Luna, L G, Coady, K, McFadden, JR, et al. (2013). *J. Anal. Toxicol*, 37, 326–336.
    - Hornung MW, Kosian PA, Haselman JT, et al. (2015). *Toxicol. Sci*. 146: 254-264.
  - **Hormone analysis methods also applied to fetal and neonate rats**
    - Hassan I, El-Masri H., Kosian PA et al. 2017. *Toxicol. Sci.* 160:57-73. TH in fetal serum
    - O'Shaghnessy KL, Wood CR, Ford RL et al. 2018. *Toxicol. Sci.* 166: 163-179. Fetal neonate rat brain and serum



Summary

- Nearly two dozen MIEs have been identified for the thyroid axis, about half have high-throughput screening assay available or being developed.

- Future efforts needed to translate *in vitro* activity to *in vivo* responses to verify MIE AOP.

- Incorporate MIE and AOP into framework for use in risk assessment for chemical disruption of thyroid hormones
  - prioritization near-term goal
  - ultimately inform & develop qAOP to predict toxicity

THANK YOU