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The thyroid gland, its relevance in toxicology and the ED GD Appendix A

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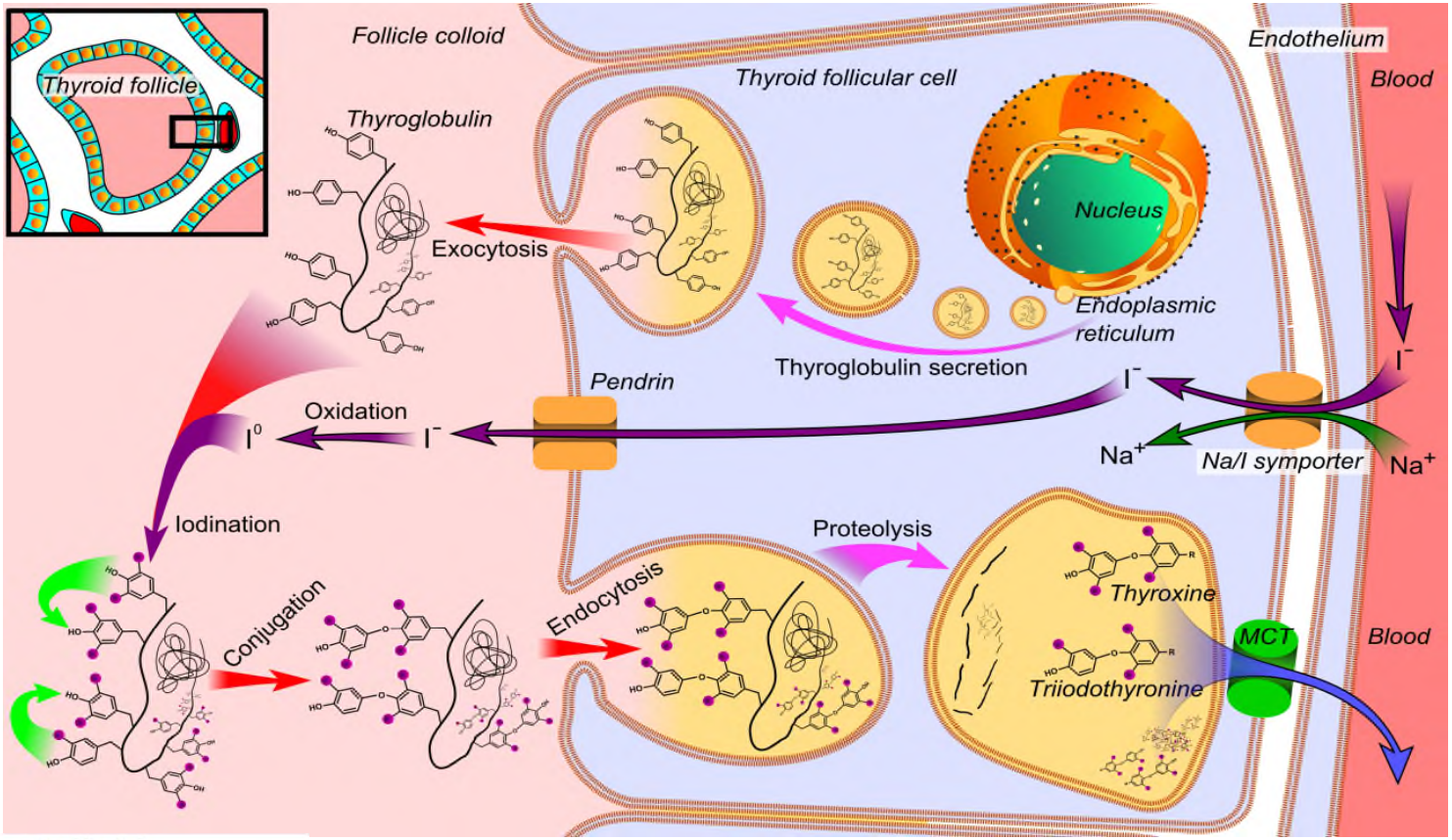
The thyroid gland

- The thyroid gland is the largest of the endocrine glands that function exclusively as endocrine gland.
- In most of the animal species and human occurs as two lobes on the lateral surface of the trachea.
- The basic histological structure is unique among endocrine glands and consists of follicles of varying size (20 to 250 µm) that contain colloid produced by the follicular cells.

The thyroid gland

- The synthesis of thyroid hormones (THs) is unique because the final assembly of hormones occurs extracellularly within the follicular lumen (FL).
- FCs trap essential raw materials (iodide) from the blood by a sodium-iodide symporter (NIS) located on the basolateral membrane and transport them rapidly against a concentration gradient to the lumen.
- In the lumen, iodine is oxidised by thyroperoxidase (TPO) in the microvilli to reactive iodine.
- The assembly of the THs in the FL is made possible by a unique protein called thyroglobulin.



Follicular Epithelial Cell:

1. **Makes/Secretes Thyroglobulin (TG) and TPO**

2. **Sequesters Iodine from blood (NIS) and transports it to colloid**

3. **Transfer TH back from colloid to secrete TH to blood**

Colloid:

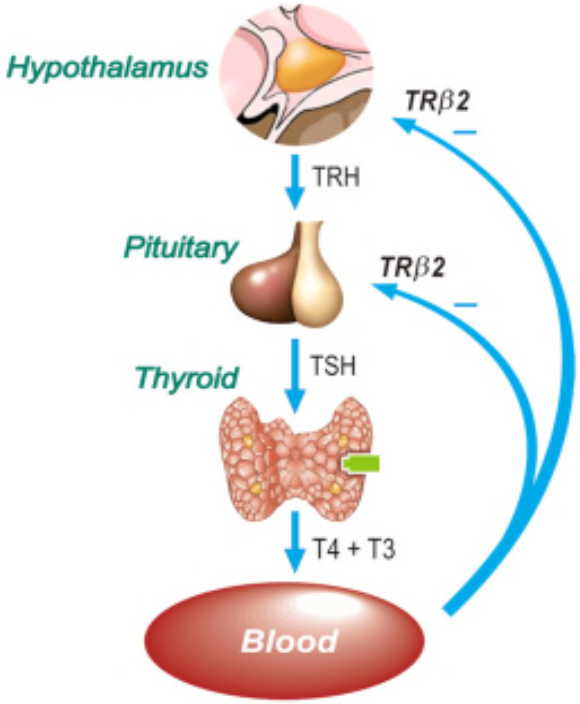
1. **Oxidizes iodine (I^- to I^0)**

2. **TPO in colloid adds I^0 to TG to form TH**

3. **Stores Iodine and TH**

All these processes are under the control of TSH. Changes in the FCs morphology are secondary to decrease in circulating THs and TSH stimulation; **however, decrements in THs can be not associated with histological changes**

HPT Axis Regulatory Feedback Loop



- T4 is released from gland to the blood in much higher quantities than T3
- Blood TH levels are controlled through negative feedback at hypothalamus and pituitary
- Pituitary release of TSH stimulates gland to produce more TH
- In clinic serum TSH, T4, T3 used to diagnose thyroid disease

Role of Thyroid Hormones in The Body Lifestage Matters!

- TH regulate diverse physiological processes in the body:

•**Adult**: Controls Metabolic rate, thermogenesis.

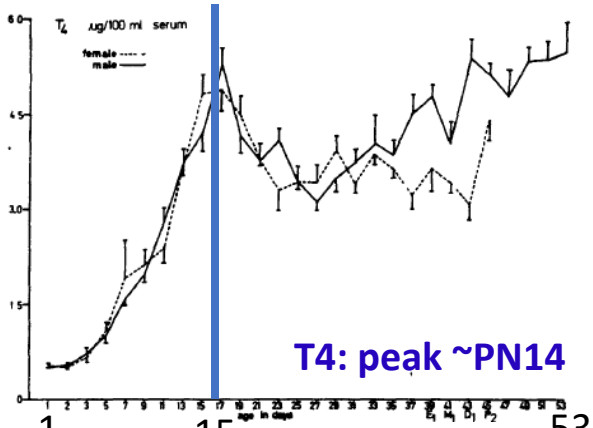
Adult - Reversible!

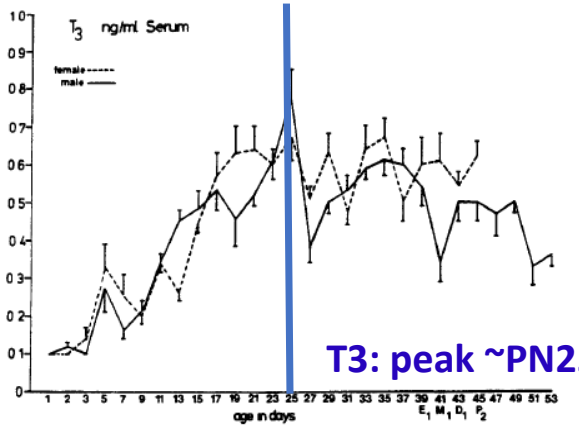
•**Fetus, Newborn, Child**: growth hormone so mediates many aspects of somatic growth and development

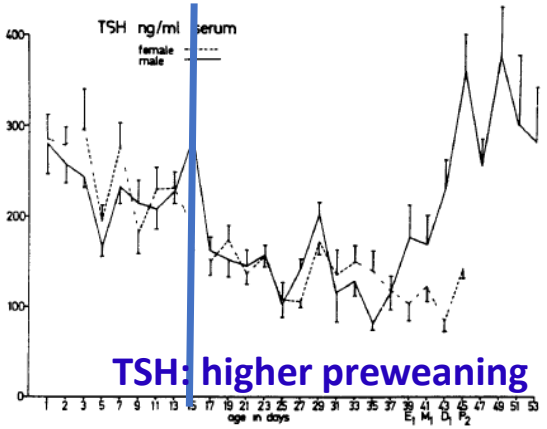
Fetus/Neonate - Permanent!

•***Especially critical for nervous system development***

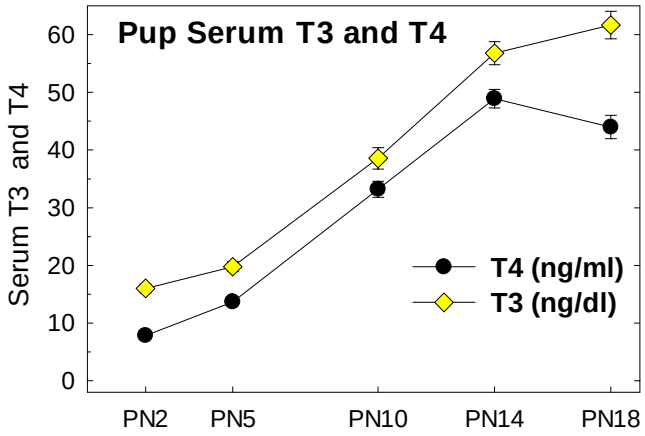
Serum Hormones Change Over Development



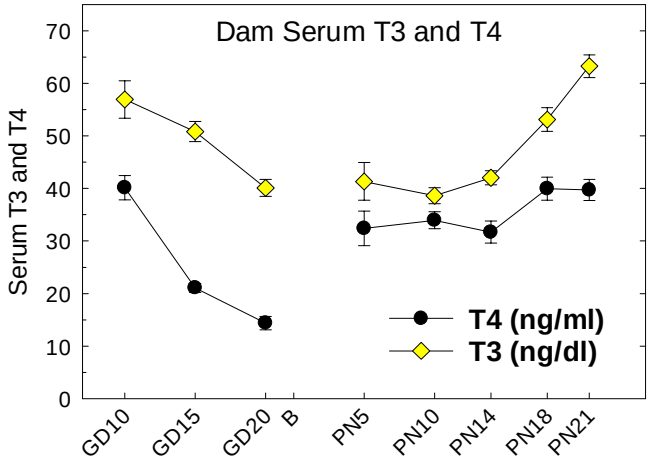




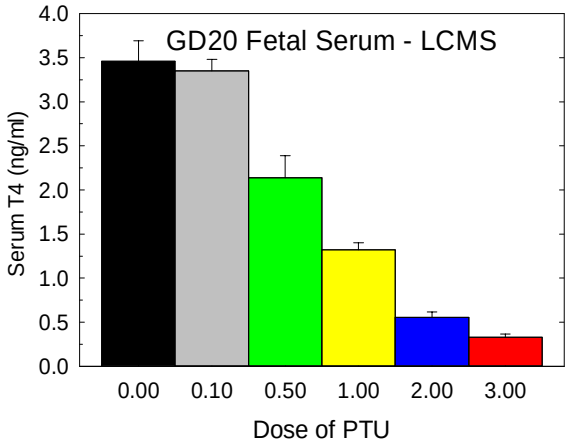
From Dohler (1979)



TH increases with age in neonate



TH drops during pregnancy



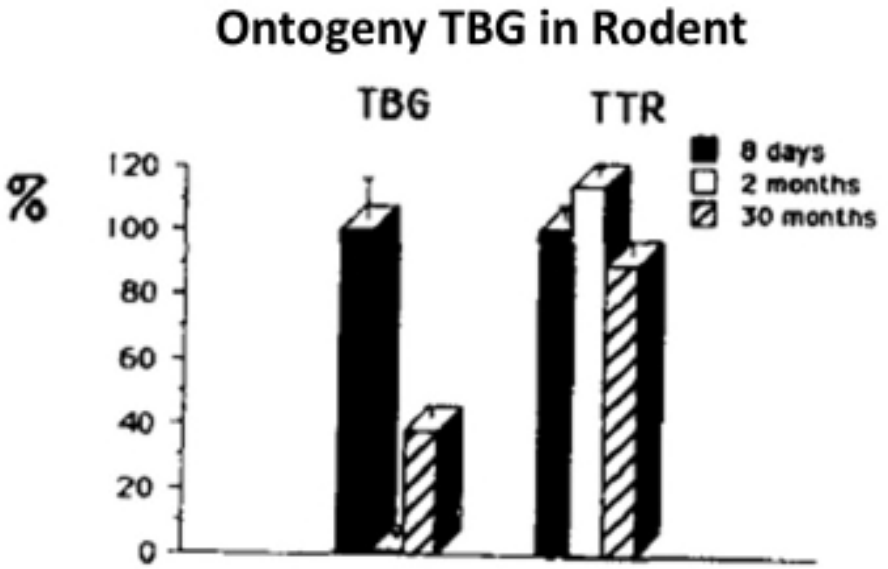
TH – low levels in fetus

From Hassan et al., Tox Sci 2017; O'Shaughnessy et al., Tox Sci, 2018

Serum Binding or Distributor Proteins

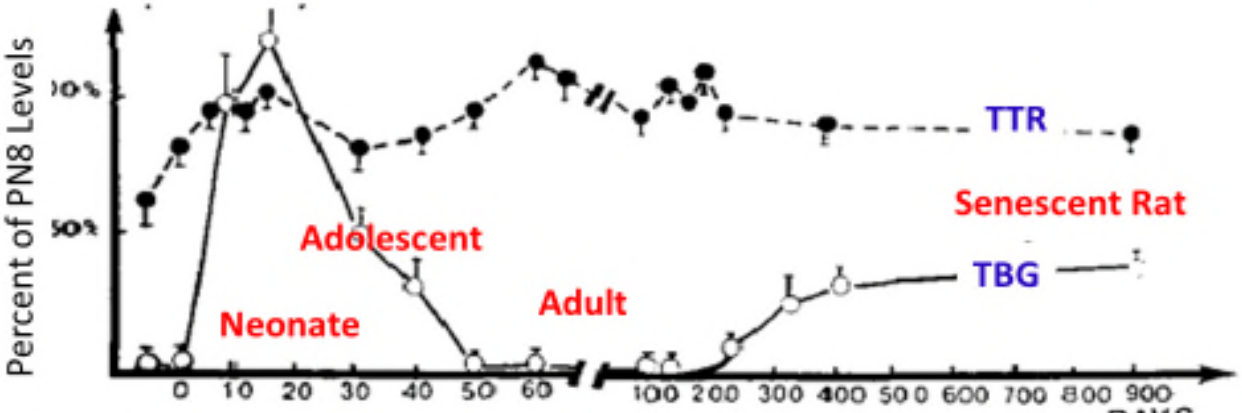
Most hormone is bound to 'distributor' proteins in the serum. Only unbound *'free'* hormones are available for uptake into tissue.

- **Thyroxine Binding Globulin (TBG):** High affinity but very few sites to carry TH
- **Transthyretin (TTR):** Lower affinity but multiple carrier sites
- **Albumin:** Most abundant but lowest affinity

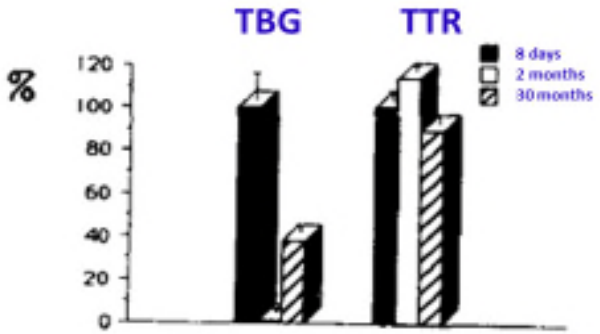


Primary binding proteins in humans and neonatal rat

TBG Primary Carrier in Human and in Young Rat.



From Savu et al., 1991, Biochimica et Biophysica Acta.

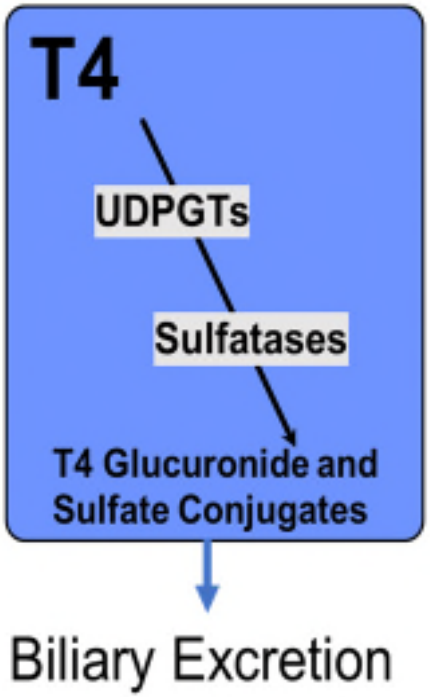


- TBG is primary TH binding protein in humans, very high affinity for T3 and T4
- Infant rodents also have TBG in serum and most T4 is bound to this molecule *(due to higher affinity);* TBG *not* present in adult rat, but it is high in the neonate
- TTR rise postnatally then constant over lifespan

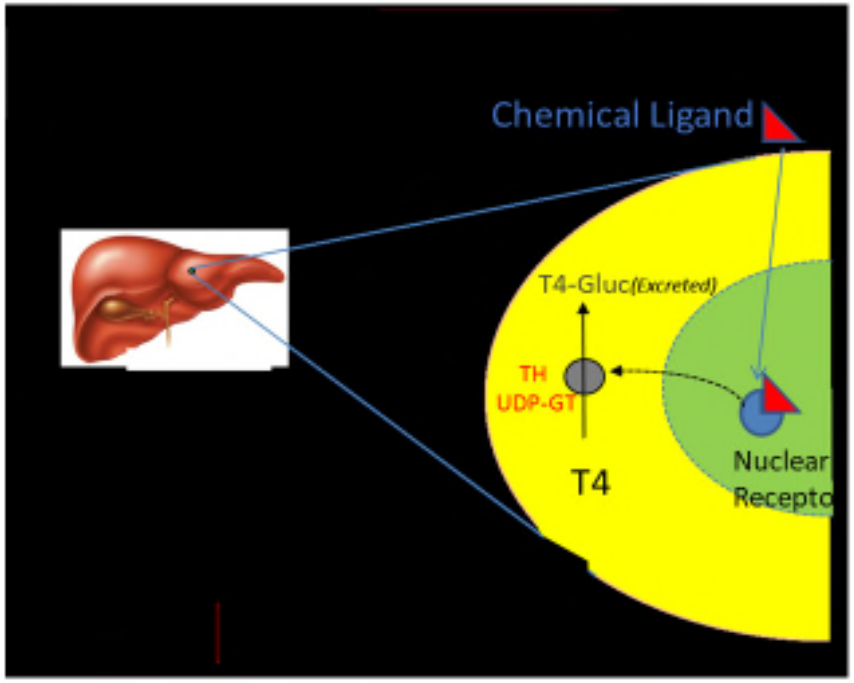
This species-difference often highlighted but based on adult only data.

Liver Metabolism of Thyroid Hormone

Systemic TH Metabolism/ Elimination

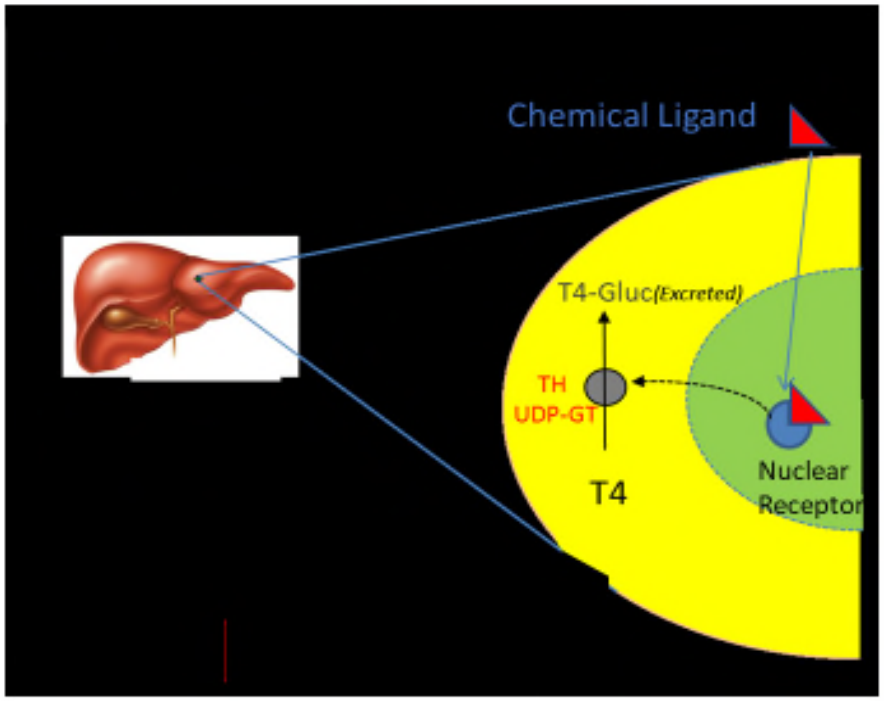


Xenoreceptors: CAR, PXR are hepatic nuclear receptors that activate glucuronide and sulfatase liver enzymes to remove chemicals from body



- TH clearance in liver is related to free TH in blood
- Activation of these receptors also clears TH

Hepatic Clearance of Thyroid Hormones



- TH clearance in liver is related to free TH in blood
- "Xenosensors" (e.g., CAR, PXR) are hepatic nuclear receptors that activate glucuronide and sulfatase liver enzymes to remove chemicals from body
- Activation of these receptors also clears TH

- Humans may be less sensitive to glucuronidation/clearance than rodents
- Species differences in chemical induction of CAR/PXR activation

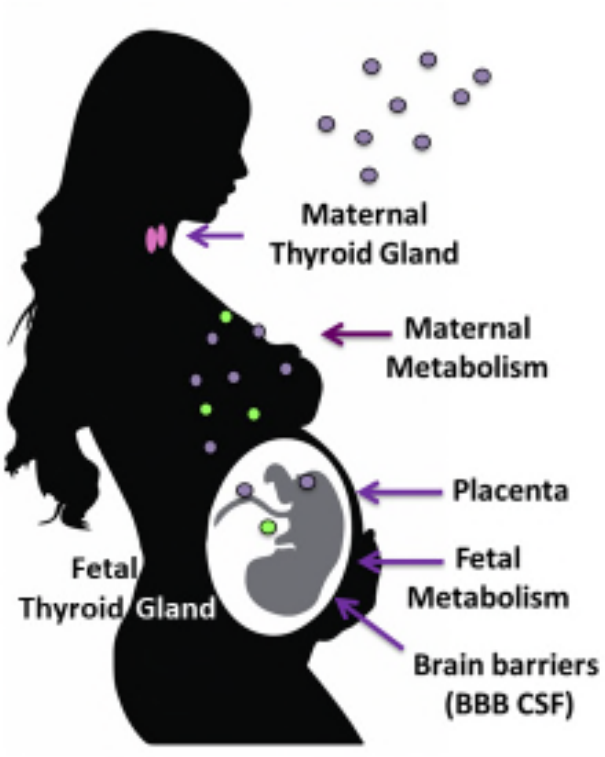
Ennulat et al. 2010: decrease in serum T4 in volunteers given rifampicin alone or with phenobarbital
Larsen et al. 1970: decrease in serum T4 following treatment with diphenylhydantoin

Brain TH and TH Action

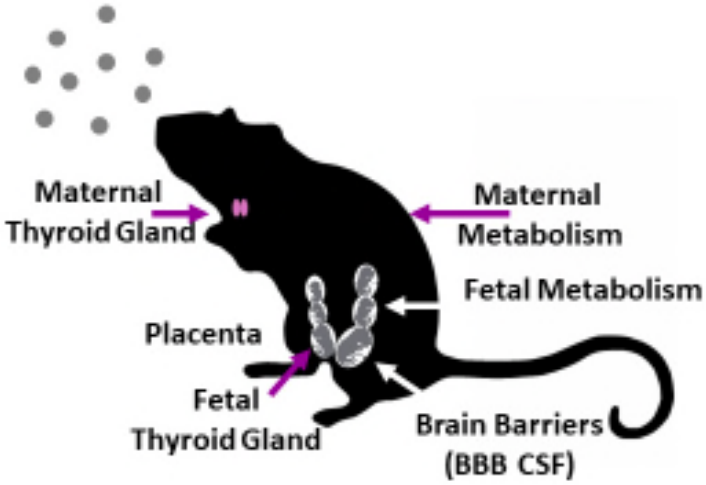
- Primary action ofT3 is to Regulate Transcription of Genes that Modulate Brain Development
- Coordinated Temporal and Spatial Control of TH Action is Essential
- Many Genes Modulate Brain Development –
  - Limited Dose-Response Data Available, largely limited to model chemicals – PTU, MMI
  - Limited data on temporal and spatial resolution (*O'Shaughnessy et al., 2018*)
- There is no argument that adequate supplies of TH are essential for normal development.
- Severity of neurological outcome is dictated by timing, duration, magnitude of perturbation.
- Effects of More Subtle Perturbations of TH Action ?

Rodent Models Mimic The Complexities of Thyroid Biology During Development

Human Pregnancy Thyroid and Brain



Rodent Models Thyroid and Brain



Most of what we know has derived from Rodent Models
Basic Components of Thyroid Synthesis and Regulation
Ontogeny of Thyroid Function in Fetus/Neonate
Metabolism Mom and Fetus
Presence of Placental and Brain Barriers
Rodent models parallel human neurological phenotype

Artwork compliments of K. O'Shaughnessy

Rats ≠ Humans - Species Differences Exist

1. T3:T4 ratios from gland vs deiodination
2. Serum TH half-life and gland storage capacity
3. Sensitivity of rodent to TSH-mediated hypertrophy of gland
4. Hepatic Metabolism – 1° site of action of many pesticides
5. Fidelity and Redundancy of TH Transporter Proteins
6. Timing of Brain Development

Uncertainties in the histopathological assessment

- Because sustained TSH increases thyroid cell hypertrophy and hyperplasia, thyroid histopathology has become a standard end point for thyroid toxicity studies.
- There is a general concept that FC hyperplasia is adverse (increase the risk of thyroid cancer), but thyroid hypertrophy is a physiological adaptation that maintains homeostasis.
- Concept limitations:
  - Changes in the thyroid histopathology are not a direct measure of changes in thyroid hormone level.
  - They are rather a measure in TSH level.
  - TSH is a direct measure of THs action but is usually ignored as surrogate of TH action on all TH-responsive genes
  - Thyroid histopathology can be a sophisticated measured endpoint (computer assisted morphometric analysis assisted by statistical analysis) or can be a less formal non-parametric assessment resulting in very different results.
  - An important marker of TH is being ignored. There is ample evidence that circulating levels of TSH is a valid marker of TH action and is therefore expected other TH-responsive genes to be affected in similar way.
- These considerations made the assumption of "adaptive change" as uncertain

Uncertainties in TH assessment

- In the OECD and EPA guideline studies, samples are collected at different time points or life stages within the protocol.
- Methods may be not adequate for the smaller sample volume per animal and lower concentration of TH and TSH in foetuses and pups.
- Different sources of variation for TH and TSH exist:
  - Physiological
  - Procedural
  - Artefactual
  - Quantitative

TH measurement, potential sources of variability

- Physiological:
  - Differences in age, sex, strain, diet, fasted vs. non fasted, stress (i.e method of blood collection), and time of blood collection (i.e. diurnal variation of hormone concentrations).
- Procedural:
  - Order of sample collection (random or counterbalanced vs. group order), blood collection site and technique and anaesthesia.
- Artifactual:
  - Poor quality of sample (inappropriate anticoagulant, storage conditions or handling)
- Quantitative:
  - Differences in quantitative methods

Analytical variation

- Established assays:
  - Manually run immunoassays (RIA)
  - Microtiter plate enzyme-like immunoassays (MTP-EIA)
  - Automated assays are also used but require samples of higher volume
  - LC-MS or UPLC-MS/MS are representing a methodological improvement but require more experienced technicians, longer measurement times and higher instruments costs
- Challenging exists for adapting commercial immunoassays kits and a carefully designed strategy for validation of immunoassays is crucial for establishing parallelism of matrix samples to the reference standard, assay sensitivity, specificity and precision.

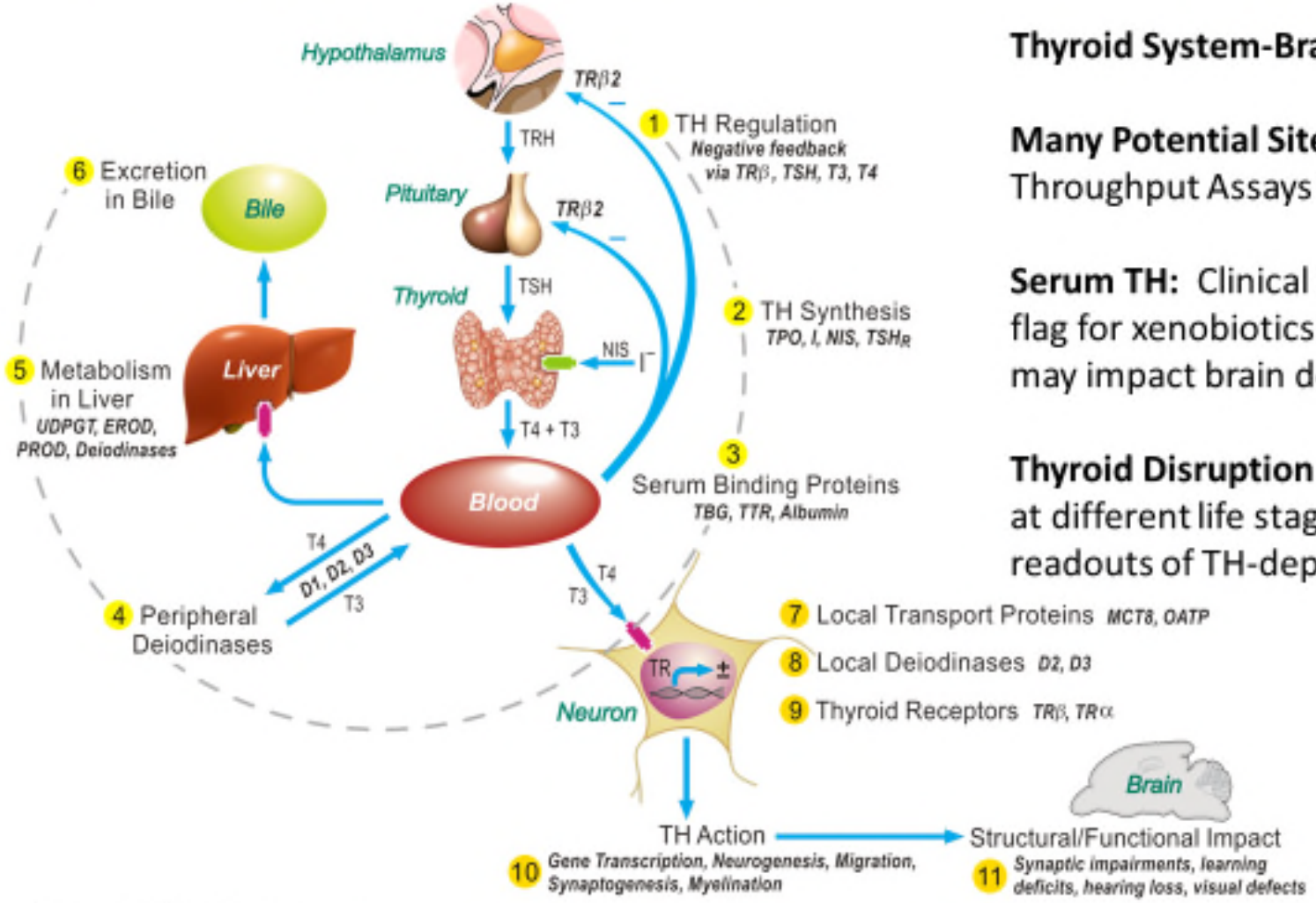
HCD for thyroid hormone assays

To obtain an insight regarding TH hormones measurements and use of HCD, the following are relevant considerations

- Age
- Strain
- Methods
- Study design
- Sample type
- Time of sampling
- Method of sacrifice
- Use of anaesthesia
- Fasting state
- LOD and LOQ defined by the laboratory

- Range of CVs for control groups with the median and mean CV, the average of study mean and the LOQ for T3, T4 and TSH at different ages.
- Reference to the validation study or positive control

Challenges in Assessing Risk Thyroid Disrupting Chemicals



Gilbert et al., 2011, Neurotoxicology
Adapted from Boas et al., 2006

Thyroid System-Brain Development- It is Complex!!

Many Potential Sites of Chemical Disruption: High Throughput Assays Developed to Target These Sites

Serum TH: Clinical tool to diagnose thyroid disease; flag for xenobiotics that disrupt thyroid signaling and may impact brain dev't

Thyroid Disruption and Brain - Different consequences at different life stages; absence of simple 'brain-based' readouts of TH-dependent disruption

Appendix A

- The basic principle of the ED guidance is hazard base and for the T modality the following are the main elements to consider:

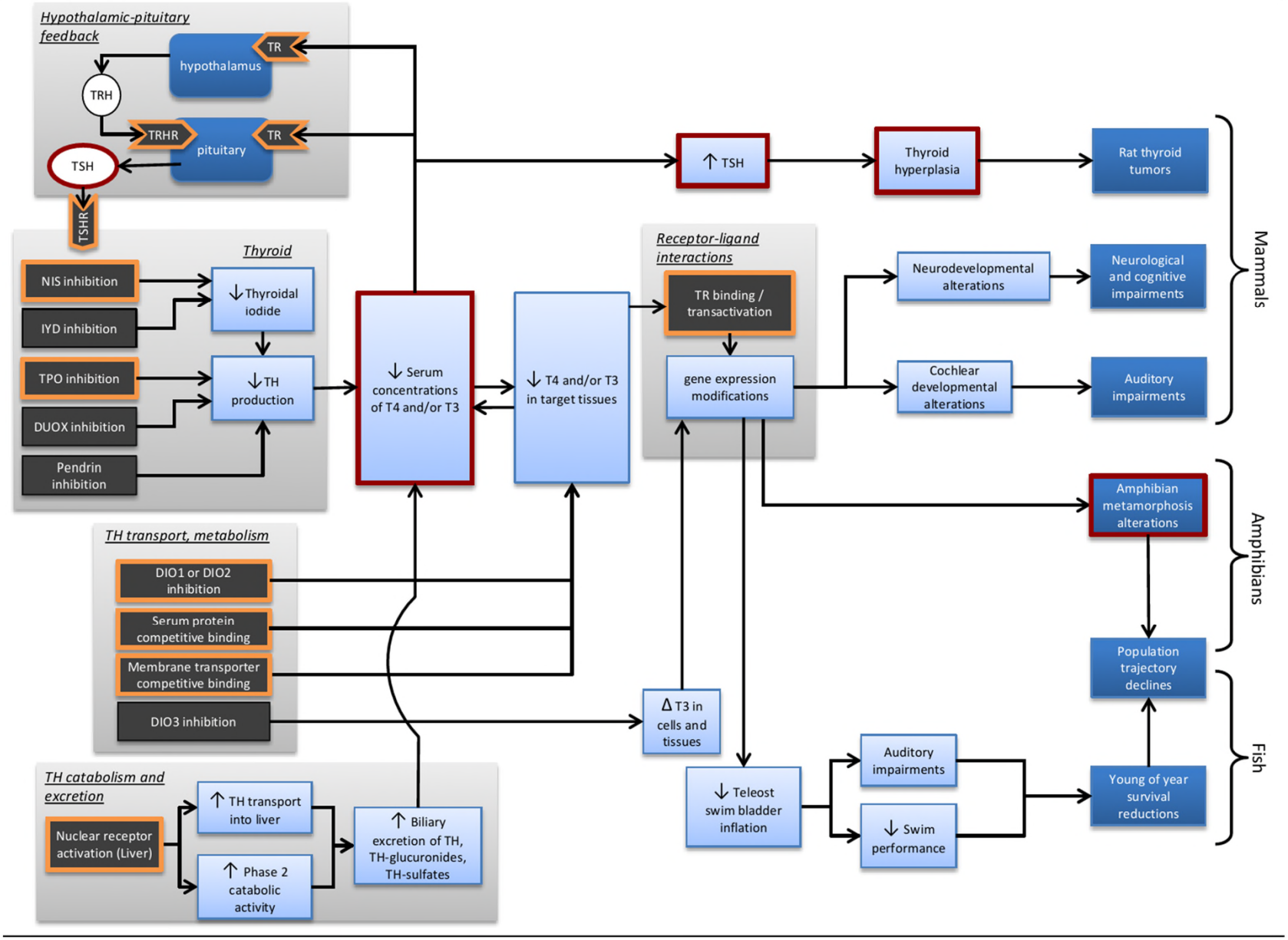
  1) Substances inducing histopathological changes in the thyroid, **with or without changes in the circulating levels of THs**, would pose a hazard for human thyroid hormone insufficiency in adults as well as pre- and post-natal neurological development of offspring.

  2) Substances that alter the circulating levels of T3 and/or T4 **without histopathological** findings would still present a potential concern for neurodevelopment.

  3) In the absence of substance-specific data which provide proof of the contrary, humans and rodents are considered to be equally sensitive to thyroid-disruption (including cases where liver enzyme induction is responsible for increased TH clearance).

Appendix A

- A MOA should follow, based on data and/or coherence analysis as described in the EFSA/ECHA ED guidance.
- Demonstration of the principal MOA is applicant responsibility; however the Appendix A in the GD indicates a sensitive approach based on the current knowledge for the most common chemically induced molecular initiating events.
- The human relevance should be substantiated by the WOE analysis of all evidence with the inclusion of human data.


Hypothalamic-pituitary feedback
hypothalamus
TR
TRH
TRHR
pituitary
TR
TSH
TSHR
Thyroid
NIS inhibition
IYD inhibition
TPO inhibition
DUOX inhibition
Pendrin inhibition
↓Thyroidal iodide
↓TH production
↓ Serum concentrations of T4 and/or T3
↓ T4 and/or T3 in target tissues
Receptor-ligand interactions
TR binding / transactivation
gene expression modifications
↑ TSH
Thyroid hyperplasia
Rat thyroid tumors
Neurodevelopmental alterations
Neurological and cognitive impairments
Cochlear developmental alterations
Auditory impairments
Mammals
Amphibian metamorphosis alterations
Amphibians
Population trajectory declines
TH transport, metabolism
DIO1 or DIO2 inhibition
Serum protein competitive binding
Membrane transporter competitive binding
DIO3 inhibition
Δ T3 in cells and tissues
↓ Teleost swim bladder inflation
Auditory impairments
↓ Swim performance
Young of year survival reductions
Fish
TH catabolism and excretion
Nuclear receptor activation (Liver)
↑ TH transport into liver
↑ Phase 2 catabolic activity
↑ Biliary excretion of TH, TH-glucuronides, TH-sulfates

Appendix A

- Also note, that the ECHA/EFSA ED GD is not mentioning any MOA as not human relevant for endocrine disruption and that a considerable effort is expected to support the non-human relevance for endocrine mediate adverse effects (e.g. changes in thyroid histopathology).
- There is evidence that chemical agents capable of inducing hepatic metabolism can affect T4 levels in humans (Ennulat et al. 2010)
- The EFSA/ECHA ED GD acknowledge the rat sensitivity for thyroid mediated effects.
- Therefore, a possibility is testing the substance in the most sensitive population with evaluation of sensitive endpoints that are expected to be less compromised by the physiological differences between human and rodents.

Appendix A

- Appendix A is quoting:
- Testing to investigate critical periods of development (i.e. in pregnant females, the fetus and newborn) could be conducted in place of the rat DNT study to generate mechanistic data to confirm or refute the observed change in circulating TH. (US EPA, 2005).
- This study is intended to generate specific data on the thyroid to establish the ability of a chemical to disrupt thyroid function in pregnant females and in the fetus and newborn.
- This special study is therefore expected to be conducted based on the results of a study(ies) in adult animals that provide evidence that a substance produces effects on thyroid function.
- Alternatively a DNT OECD TG 426 can be conducted and special emphasis in the study design should be given for thyroid sensitive endpoints i.e. THs and TSH assessment using a sensitive analytical methodology, thyroid histopathology and thorough evaluation of the brain.

A special thanks to Mary Gilbert from the US EPA for the permission of using her slides and to the EFSA PREV UNIT mammalian tox team for their everyday support