UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI SCIENZE DEL FARMACO

Master's degree in

Safety Assessment of Xenobiotics and Biotechnological Products

Mechanistic evaluation of rodent studies for selected thyroid disrupting chemicals (TDCs). From key events to adverse outcome pathway.

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Academic year 2020-2021

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Acknowledgements

First and foremost, I would like to show my appreciation for Prof. Marina Marinovich for all the effort she has put to create this new specialized master's degree on risk assessment and gives her best for the students to get an insight of this diverse, complicated and interesting matter. Aside from that, I acknowledge all her support as my supervisor in this thesis and her help in the cooperation with *BASF SE*, where this project took place.

Furthermore, I would like to give my special thanks to my direct supervisor of the project at *BASF SE*, Dr. Stephanie Melching Kollmuss, first for the opportunity she gave me to work in a scientific environment of high standards and with such an exciting topic. Second, I am grateful for her attendance, detailed explanations, endless support and patience throughout these months.

Likewise, I would like to show my deepest appreciation for Dr. Volker Strauss who was always there to assist and advise me with precision about everything in a tireless way.

Moreover, I want to express my gratitude for the PhD student Andreas Georg Weber for his readiness to help with any question I had and for his useful tips.

The welcoming manner, knowledge, explanations and enthusiasm of all these people made my time in this project pleasant, engrossing and kept me motivated even in the hard days of this extraordinary situation of the pandemic.

None of this would have been possible without the continuous support and encouragement of my parents throughout my education, which I sincerely appreciate. Finally, many thanks to my friends who have been next to me for every good or bad time during this journey.

Declaration of oath

I hereby certify that the work reported in this thesis is my own. The ideas taken directly or indirectly from the sources, have been clearly cited in the text as well as in the corresponding section at the end of the thesis. I have not sent the present work to any other examiner in this or other form or published this work anywhere.

(Place, Date) (Signature)

List of abbreviations

AhR	Aryl hydrocarbon Receptor
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
AP	Ammonium Perchlorate
ATD	Antithyroid Drug
BAT	Brown Adipose Tissue
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CAR	Constitutive Androstane Receptor
CNS	Central Nervous System
Cyp	Cytochrom P450
DIO	Deiodinases
DIT	Diiodotyrosine
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
GABA	Gama aminobutyric acid
GCL	Granule Cell Layer
GD	Gestation Day
GLP	Good Laboratory Practice
HPT axis	Hypothalamic-Pituitary-Thyroid axis
i.p	Intraperitoneally
ID	Iodine Deficiency
IPCS	International Program on Chemical Safety
KE	Key Event
LTP	Long Term Potentiation
MCT8	Monocarboxylate transporter 8
MIE	Molecular Initiating Event
MIT	Monoiodotyrosine
MOA	Mechanism of action
MRI	Magnetic Resonance Imaging
NIS	Sodium Iodide Symporter
NR	Nuclear Receptor
OATP2	Organic Anion Transporting Polypeptide 2
OECD	Organisation for Economic Co-operation and Development
PCB	Polychlorinated bisphenols
PCR	Polymerase Chain Reaction
PND	Postnatal day
PTU	6-Propylthiouracil
PXR	Pregnane X Receptor
rT3	reverse T3
SBH	Subcortical Band Heterotopia
SULT	Sulfotransferases
T3	3,3’,5-triiodo-L-thyronine

T4	Thyroxine
TBBPA	Tetrabromobisphenol-A
TBG	Thyroxine-binding globulin
TDC	Thyroid Disrupting Chemical
TG	Test Guidelines
TH	Thyroid Hormones
THBP	Thyroid Hormone Binding Protein
TPO	Thyroid peroxidase
TR	Thyroid hormone Receptor
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
TTR	Transthyretin
UDPGT	5'-diphospho glucuronosyltransferase
WHO	World Health Organization

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1 Introduction

1.1 Aim of the thesis

For this thesis evaluation of mammalian rodent studies of six compounds, with assumed or known thyroid modulating effects, was done. The focus was on their neurodevelopmental effects in order to develop an Adverse Outcome Pathway (AOP) regarding Molecular Initiating Events (MIE), Key Events (KE) and an Adverse Outcome (AO) of neurological dysfunction.

In humans it is now broadly accepted that a reduction in free T4 levels in serum of pregnant women in the first trimester causes neurodevelopmental changes in the offspring (Gilbert et al., 2020; Sauer et al., 2020). In epidemiological studies it was shown that in children of iodine deficient or hypothyroid pregnant women alterations in brain structure and function, psychomotor deficits, IQ and cognitive function decrease were observed (Gilbert et al., 2020). To evaluate those effects, *in vivo* studies are done, mainly using rodents as an animal model. The present thesis focusses on two aspects regarding rodent studies:

- **Do serum thyroid hormone (TH) changes in pregnant rodents provoke neurodevelopmental effects in offspring?** So, is there causality between the observed Key Events starting from TH changes resulting to brain deficits?
- **Are rodents an appropriate model for extrapolation to humans regarding neurodevelopmental effects connected to hypothyroidism?**

The substances used for this study were selected in the frame of the European Project ERGO (EndocRine Guideline Optimization), which is part of the project cluster called EURION. [That is the biggest project for endocrine disruptors in the EU for new testing and screening methods in the frame of the European Commission's Horizon 2020 Research and Innovation Programme. It consists of 8 projects with different endpoints.] ERGO focuses on thyroid disruption with one of its aims being the establishment of thyroid-related parameters across mammalian (rodent, humans) and non-mammalian vertebrate (fish and amphibians) species. For this purpose, six reference or assumed thyroid modulating compounds (carbamazepine, iopanoic acid, sodium perchlorate, 6-propylthiouracil (PTU), tetrabromobisphenol-A (TBBPA) and resorcinol) with different thyroid MIEs as well as ampicillin as a negative control were selected for running fish studies and performing literature evaluation of mammals. The thyroid related MIEs covered by ERGO and thereupon by this thesis are the following: displacement of thyroid hormones (TH) at Thyroid Hormone Binding Proteins (THBP), Thyroid Peroxidase (TPO), Sodium-Iodide-Symporter (NIS), Deiodinases I-III (DIO) inhibition, Thyroid Hormone Receptor (TR) modulation, Xenobiotic Receptor Activation.

Here it is important to mention when a substance can be considered to have endocrine disrupting properties with respect to humans (relevant for Regulation 528/2012 and 1107/2009):

(a) it shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;

(b) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;
(c) the adverse effect is a consequence of the endocrine mode of action (EU, 2017).

Adverse effects that are non-specific secondary consequences of other toxic effects shall not be considered for the identification of the substance as endocrine disruptor (EU, 2018).

Possible endocrine disruptors might target various endocrine signaling pathways like Estrogen, Androgen, Thyroid or Steroidogenesis (EATS) specific.

1.2 Thyroid background

1.2.1 Anatomy and function

The typical thyroid gland in mammals is a bilobed organ with the two lobes being connected by a bridge of thyroid tissue which lies on, and is located ventro-laterally to, the trachea. The thyroid gland is the first of the endocrine tissues to develop in the rat. It begins as a ventral down growth of endoderm from the primitive pharynx in the region of the first pharyngeal (branchial) pouch. As the thyroid gland develops, it separates from the floor of the mouth cavity by involution of the thyroglossal duct and it migrates caudally into the neck (Mense & Boorman, 2018). Also, during human development, the thyroid is located in the back of the tongue and must migrate to the front of the neck before birth.

As for the histology of the thyroid gland, each lobule contains a cluster of follicles, which are the structural and functional unit of the thyroid gland. A follicle consists of thyrocytes (follicular cells) and the colloid, which is a semi-solid substance. Follicular cells are responsible for producing thyroglobulin, which is then stored as colloid in the lumen of the follicles. In comparison to humans the follicles of rodents are smaller with less colloid and a higher production rate (see *table 2*). Another cell type that can be found in histological preparations of thyroid tissue is the parafollicular cells (also called C cells). They can be found within the basal lamina of thyroid follicles and they are a subtype of neuroendocrine cells responsible for the production of calcitonin (Crumbie, 2021).

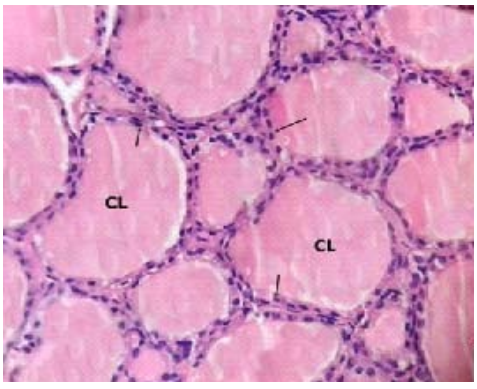

Figure 1: Thyroid gland of an untreated adult rat showing thyroid follicles lined by follicular epithelial cells containing colloid (CL)(Patil & Dhurvey, 2014) .

The thyroid produces, stores, and secretes thyroid hormones (TH) with the biologically active ones being T3 (3,3',5-triiodo-L-thyronine) and T4 (thyroxine). In humans, T4 is quantitatively the main thyroid hormone synthesized and secreted whereas T3 is the more bioactive molecule, mostly produced by deiodination of T4 in peripheral tissues. In rats the production rate of T4 is a lot higher than in humans (see *table 2*). As TH have been studied extensively in terms of their physiological and biochemical actions in different species, it has been shown that their major characteristic is the multiplicity of their actions. Studies have shown that TH receptors (TR) are highly conserved between different species and the interaction between the TR and the ligand is believed to be the primary intracellular event for gene expression. However, there are major differences that account for difficulties in effect-extrapolation between species and this diversity is generated by species- and tissue-specific factors and mechanisms (Chatterjee et al., 1997; Mangelsdorf & Evans, 1995; Tata, 1998).

Thyroid hormones are essential for the general metabolism, which means they maintain the rate at which the body utilizes fats and carbohydrates and ultimately the conversion of calories and oxygen to energy. In other words, the Hypothalamic-Pituitary- Thyroid (HPT) Axis plays a critical role in thermoregulation of the body (Johnstone et al., 2013). TH also regulate the synthesis and activity of many intracellular enzymes like Na^+/K^+ -ATPase as well as the size and activity of mitochondria. Moreover, TH are of big importance in growth and development of the brain as well as other organs in mammals in the fetal and postnatal growth (Campinho et al., 2014) .

The regulation of thyroid hormone delivery to tissues and cells for the maintenance of thyroid hormone signaling during development and in the adult represents a very complex and unique network of feedback systems. Environmental factors like iodine deficiency or the presence of specific toxicants, can perturb the HPT axis at various points of regulation. A fundamental question in this area of research is if reduction in maternal TH levels in serum also reduces the activity of TH in the offspring target organs or if this is compensated through homeostatic regulations or even locally through deiodinases at the target organ, which in the frame of this thesis is the brain.

1.2.2 Thyroid Hormone Production and Homeostasis

The thyroid gland in mammals uses thyroglobulin (Tg), a dimeric glycoprotein that contains approximately 100-120 tyrosine residues. Tg is stored in the colloid, as the starting molecule for the subsequent synthesis of the major thyroid hormones, T3 and T4. The hormone that controls the rate of thyroid hormone production within the thyroid is thyroid stimulating hormone (TSH), secreted by the thyrotrophic cells within the anterior pituitary gland, which in turn is regulated by the thyrotropin releasing hormone secreted by the hypothalamus (see *figure 2*) (Dickhoff & Darling, 1983). Binding of TSH to its receptor on the thyroid follicular cell membranes triggers the up-regulation of the sodium-iodide symporter (NIS) on the basolateral membrane of the follicular cells, resulting in the active transport of iodide from the plasma into the follicular cells and an increase in the intracellular concentrations of iodide. Once inside the cell, the iodide is oxidized by the enzyme thyroid peroxidase (TPO) to the iodinium cation, which then iodinates tyrosine residues of the thyroglobulin proteins in the follicular colloid to produce mono- and diiodotyrosine and finally links a monoiodotyrosine (MIT) molecule to a diiodotyrosine (DIT) molecule to form T3 and two diiodotyrosine molecules together to form T4. Thenceforth, thyroid hormones are internalized into

endosomes at the apical surface of the cells by endocytosis. Then the peptide linkage between the thyroid hormones and thyroglobulin is enzymatically cleaved, to release the hormones which are subsequently actively exported into the circulation, through the basolateral membrane of the follicular cells via the monocarboxylate transporter 8 (Di Cosmo et al., 2010). Once in the circulation the majority of the hormones reversibly complex with binding proteins for transport to other tissues, although a small portion remains free in the plasma.

The HPT axis is a complex and highly regulated neuroendocrine system which functions to maintain circulating T4 and T3 concentrations in the blood and target tissues within a normal range in euthyroid organisms.

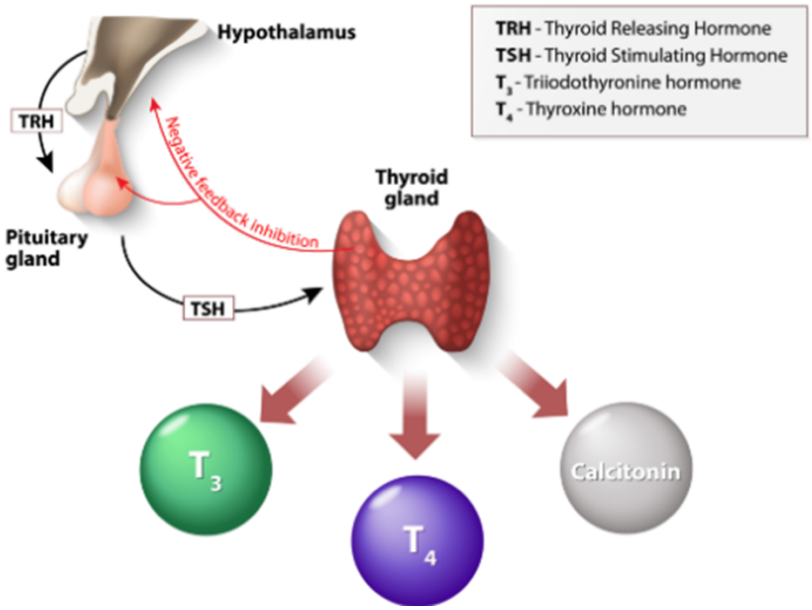

Figure 2: Thyroid hormone production through the HPT axis. (https://www.renewedvitalitymd.com/thyroid-replacement/thyroid-hormones-interact/)

1.2.3 TH importance for human pathology

With alterations in thyroid hormones and abnormal thyroid growth, thyroidal pathophysiological changes can occur. Some of the pathologies that are connected to the HPT axis in humans are:

Hyperthyroidism is the condition that occurs due to excessive production of thyroid hormones by the thyroid gland. People with hyperthyroidism are often sensitive to heat, hyperactive, and eat excessively. Goiter (a bulge in the neck) is sometimes a side effect of hyperthyroidism. This is due to an over-stimulated thyroid and inflamed tissues.

Hypothyroidism is the condition that occurs due to insufficient production of thyroid hormones by the thyroid gland. A hypothyroid adult may experience sensitivity to cold, little appetite, and overall sluggishness.

Developmental toxicity. A change in thyroid hormones during pregnancy or in early child development can cause cretinism. This disorder has two main types: neurological and myxodematous or hypothyroid cretinism. Neurological cretinism is associated with severe

iodine deficiency (ID). It is the most common and more severe form and is characterized by severe mental retardation, deaf mutism, spastic diplegia, stance and walking disorders in the child. Myxodematous or hypothyroid cretinism is associated with severe hypothyroidism, which was caused by iodine deficiency. This type is characterized by mental retardation (less severe than in neurological cretinism), dwarfism and hypothyroidism with associated physical symptoms e.g. coarse and dry skin, husky voice, delayed sexual maturation, delayed reflexes. In contrast to neurological cretinism these individuals usually have severe hypothyroidism. Neurological cretinism is the result of affected early gestation, while hypothyroid cretinism is the result of affected later gestation, which leads to the differences in symptoms (Kouzmina, 2012).

Table 1: Comparison of the effects for neurological and hypothyroid cretinism (Kouzmina, 2012).

Features	**Neurological cretinism**	**Hypothyroid cretinism**
Mental retardation	Present, often severe	Present, less severe
Deaf mutism	Usually present	Absent
Cerebral diplegia	Often present	Absent
Squint	Often present	Absent
Stature	Usually normal	Severe growth retardation usual
General feature	No physical signs of hypothyroidism	Coarse dry skin, husky voice
Reflexes	Excessively brisk	Delayed relaxation
Electrocardiography	Normal	Small voltages QRS complexes and other abnormalities of hypothyroidism
X-ray limbs	Normal	Epiphyseal dysgenesis
Effect of thyroid hormones	No effect	Epiphyseal dysgenesis improvement

Moreover, children with an iodine deficiency in utero or early postnatal period may be diagnosed with a developmental disorder like learning disabilities, autism, attention deficit or hyperactivity disorder (ADHD). This seems to be due to anatomical and functional changes in the brain like reduced synaptogenesis, myelination, cortical dysplasia etc. especially in the first half of pregnancy which is important for the development of the neocortex and the cortical cell migration (Berbel et al., 2007). Koreevar et al. (2016) has shown an association between both low and high maternal free thyroxine concentration during pregnancy with lower child IQ, lower grey matter and cortex volume. Moreover, in terms of structural changes in the brain De Escobar et al. (2007) used non-invasive techniques such as computerized tomography or

magnetic resonance imaging (MRI) on adult cretins which showed widespread atrophy of the cerebral cortex and subcortical structures of the pons and mesencephalon with corresponding enlargement of the different cerebral cortex areas. Also, results obtained from aborted fetuses in an area of China where severe ID and cretinism occur, have suggested that prenatal ID exhibits certainly a depressive effect on the brain development in some of the fetuses in those areas (Jia-Liu et al., 1989).

Thyroid cancer is the ninth cancer in terms of incidence worldwide, with about 430'000 cases in women (10.2/100'000 with a mortality of 0.4 of the cases) and 131'000 in men (3.1/100'000 with a mortality of 0.5 of the cases) in 2018 (La Vecchia et al., 2021). Incidence rates have been steadily increasing over the past few decades, particularly in women. High incidence areas are Japan and the Pacific Islands, Italy and several countries in the Americas. If recent trends continue, thyroid cancer will be the fourth most common cancer in the U.S. by 2030. Approximately two/thirds of all thyroid cancer are papillary carcinomas. Other subtypes include follicular carcinomas (10-20%), medullary carcinomas (5-10%) and anaplastic carcinomas (<5%) with the last one accounting for a large portion of the mortality because of its poor prognosis. Although the increasing screening and diagnostic testing is likely the major contributor to the rising incidence of thyroid cancer some other major suspected risk factors include exposure to ionizing radiation during childhood, benign thyroid disease, iodine imbalance, and familial and genetic factors (La Vecchia et al., 2021; Zimmermann & Galetti, 2015).

1.2.4 Endpoints of thyroid hormone disruption

Thyroid disrupting chemicals can either act directly on the HPT axis, affecting the thyroid hormone synthesis and TH distribution via the blood or indirectly by reducing thyroid hormones in the blood by increased metabolic breakdown. In other words by disrupting the central metabolism and excretion of TH, cellular uptake by selective TH transporters of the cell membrane and intracellular activation or inactivation of TH (Noyes et al., 2019).

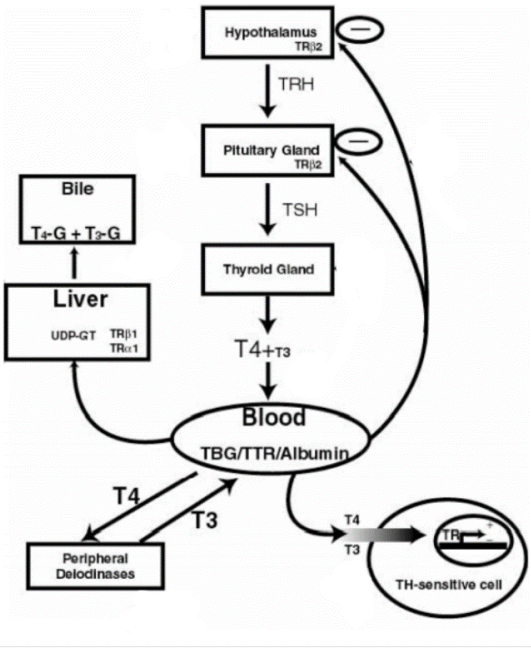

Figure 3. The mammalian hypothalamic-pituitary-thyroid axis. (Zoeller et al., 2007)

Central regulation.

The complex control mechanism of the HPT axis for thyroid gland function and growth is strongly regulated by thyrotropin releasing hormone (TRH), which is synthesized by neurons in the paraventricular nucleus of the hypothalamus. TRH affects not only the production of TSH but also its glycosylation pattern and so its biological activity. The neuroendocrine control of thyroidal TH synthesis and secretion is very sensitive to negative feedback of TH at the level of the pituitary and the hypothalamus (see *figure 3*). Several drugs (e.g. rexinoids (RXR-selective retinoids), glucocorticoids and dopamine agonists) have been shown to suppress TSH levels and cause central hypothyroidism in humans as well as in experimental rodent models (Haugen, 2009; Sharma et al., 2006; Zatelli et al., 2010).

TH synthesis & secretion

In response to TSH stimulation, the thyroid tissue produces and releases T4 and to a lesser extent T3. Since iodide is essential for thyroid hormone synthesis its uptake from the bloodstream is a critical step in this process. This is facilitated through the **sodium-iodide (Na^+/I^-) symporter (NIS),** a membrane glycoprotein located on the basolateral side of thyroid follicular cells. This symporter can be competitively inhibited or blocked by chemicals, like ions with similar size to iodide (e.g. perchlorate) and so block the active transport of iodide into the thyroid. NIS-mediated uptake of Iodide (I^-) as the first step in the biosynthesis of thyroid hormones is highly conserved across species, and the organization of the genomic sequence of human and rat NIS is highly homologous (Smanik et al., 1996). However, *in vitro* studies provide some indication for quantitative differences in NIS activity, since rat and mouse NIS proved to be more efficient in mediating I^- uptake than human NIS (Dayem et al., 2008; Heltemes et al., 2003).

After iodide is exported into the follicular colloid lumen, its organification takes place at the apical membrane of thyrocytes through the enzyme **thyroid peroxidase (TPO).** TPO catalyzes the iodination of tyrosyl residues in the thyroglobulin polypeptide chain to produce the TH precursors MIT and DIT and subsequently couples them via an ether bond to build T3 and T4. TPO is a heme-containing enzyme with two substrate sites that undergo a one-electron oxidation resulting in a two-step mechanism of iodination. It has no catalytic activity without the presence of hydrogen peroxide (H_2O_2). There is a number of chemicals that directly interfere with TPO activity and even cause an almost complete inhibition of TH synthesis, which shows the crucial role that TPO has in this process. Examples of these chemicals are the substances of PTU, methimazole and pronamide (Gilbert, 2011; Noyes, 2019).

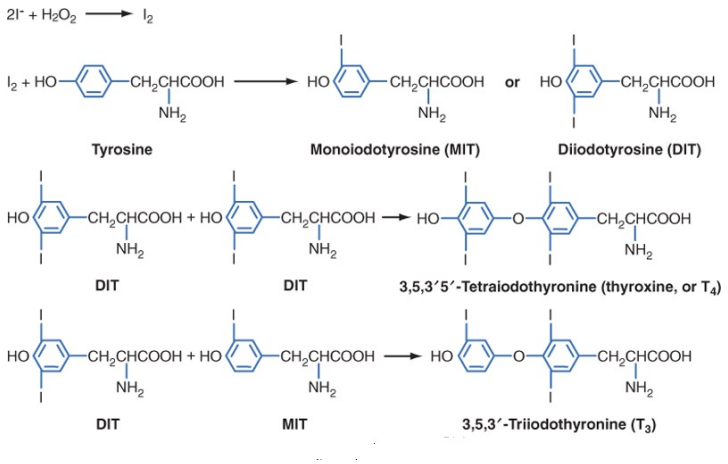

Figure 4. Chemicals reactions for the thyroid hormone synthesis. (https://www.biosciencenotes.com/synthesis-of-thyroid-hormones)

Systemic distribution and transport

Synthesized in the thyroid gland, the TH are then exported from the thyrocytes through specialized TH transporters (e.g. MCT8) across the basolateral thyrocyte membrane into the circulation. In the blood of humans approximately 0.03% of T4 and 0.3% of T3 are free (Bartsch et al., 2018). However, the majority of thyroid hormones in the blood are bound by the transport plasma proteins. In humans about 75% of T4 is bound to thyroxine-binding globulin **(TBG)** with very high affinity, 15% is bound to transthyretin **(TTR)** and the rest to albumin. The latter is very abundant but the binding to this protein is non-specific and with low affinity. In comparison to humans, adult rodents differ in their serum transport of thyroid hormones. Although there is a functional TBG gene present and the protein is expressed in reasonable levels in juvenile rats, TBG protein is present at negligible levels in the plasma of adult rats. That means that the plasma transport proteins for T4 in adult rodents are TTR and albumin, while for T3 it is predominantly albumin (Foster et al.). Even though both TTR and albumin have high capacity for binding the thyroid hormones, the dissociation rates for the two thyroid hormones are high since their affinity to the serum proteins is low. That increases the concentration of free hormones in the blood, which makes them more susceptible to metabolism and excretion and explain the much shorter half-lives of the hormones in rodents in comparison to humans (see *table 2*). TTR is synthesized in liver, brain, pancreas, retina and

placenta and is involved in the transport of T4 and retinol across the blood-brain-barrier (BBB) and to the fetus from the placenta. Many chemicals can have TTR as a target since they show high affinity to it, which might be even higher than the affinity that the endogenous ligand T4 has. Therefore, a displacement of T4 and a binding of the xenobiotic could take place (Murk et al., 2013). Some known TTR binding chemicals are tetrabromobisphenol-A (TBBPA), dioxins, OH-PCBs etc. (Hedge et al., 2009; Hallgren and Darnerud, 2002; Cheek et al., 1999, Hamers et al., 2006; Lans et al., 1994).

Metabolism & excretion

For the activation, inactivation and excretion of TH from the body there are three main pathways, namely deiodination catalyzed by iodothyronine deiodinases (DIO), sulfation by sulfotransferases (SULT) and glucuronidation by Uridine 5'-diphospho glucuronosyltransferases (UDPGT). SULTs and UDPGTs are phase-II enzymes conjugating the phenolic hydroxyl group of iodothyronines with sulfate for an inactivation of TH, and glucuronic acid for an excretion in the bile respectively. In the rat both SULTs and UDPGTs are responsible for the excretion of TH with major sites of this process being the liver and the kidney. Sulfation initiates its degradation, allowing reutilization of the iodide for the *de novo* thyroid hormone synthesis while glucuronidation appears to facilitate the fecal excretion of thyroid hormones (Foster et al., 2021). Deiodination plays an important role in the metabolism of THs and is qualitatively similar in animals and humans (Bartsch et al., 2018). Sulfation of T3 and T4 in rats and mice is less than in humans, whereas glucuronidation of the thyroid hormones predominates in rats, which causes a much higher percentage of T4 in bile than that in humans (see *table 2*) (Bartsch et al., 2018). The UDPGT enzymes are localized intracellularly within the endoplasmic reticulum while the SULTs are cytosolic enzymes. The deiodinases are localized either in the inner plasma membrane or in the endoplasmic reticulum dependent on the deiodinase type and its expression location (Köhrle, 2000).
Thyroid disrupting chemicals (TDCs) can interfere with those enzymes important for metabolism and excretion of TH. Those chemicals can block the activity or the production of these enzymes by inhibiting or inducing their expression. Some xenobiotic nuclear receptors that regulate this expression are CAR, PXR, PPAR, AhR that can be activated by several xenobiotics. For example, activators of CAR or PXR are known to upregulate transcription of a wide variety of Phase I-III enzymes, which increase hepatic catabolism and sometimes bioactivation of pharmaceuticals and endogenous thyroid hormones (Murk et al., 2013). More specifically, phase I enzymes catalyze oxidation, reduction and hydrolysis reactions. Phase II enzymes catalyze conjugation reactions for the better metabolization of molecules making them more water soluble. Finally phase III transporters for example P-glycoprotein and organic anion transporting polypeptide 2 (OATP2) are expressed in many tissues like liver, intestine, kidney and brain and play a crucial role in drug absorption, distribution, and excretion (Xu et al., 2005).

Thyroid hormone action on target organs/cellular responses

Some other molecules that are critical for the regulation of circulating TH concentrations are some cellular transporters like OATPs, MCT and multi-drug efflux transporters and are therefore also targets for endocrine disrupting chemicals.

The main hormone secreted by the thyroid gland is T4, that is deiodinated to T3, which is the most biologically active molecule. For the process of deiodination, three enzymes are responsible **(DIO1, DIO2, DIO3)** that yield the compounds T3, reverse T3 (rT3), T2 and T1, which have different biological activities. DIO1 is expressed mainly in the liver, the kidneys and the thyroid and is mostly active in the deiodination of T4 to rT3 and rT3 to 3,3'-diiodothyronine, which are inactive molecules and therefore DIO1 is mainly responsible for inactivation of TH. DIO2 is expressed primarily in the brain, anterior pituitary, brown adipose tissue (BAT), thyroid and skeletal muscle. DIO2 plays an important role in the local production of T3 from T4 in brain, pituitary and BAT. DIO2 activity is negatively regulated by TH at the posttranslational level (Gereben et al., 2008). Although that enzyme is expressed in high levels in human thyroid it is not expressed in normal rat or mouse thyroid (Wagner et al., 2003) (*table 2*) . The consequence of that lack of expression in rat is that intracellular conversion of T4 to T3 will not occur within the follicular cells. That is possibly the result of the fact that rat follicular cells are normally in a higher state of enzyme production for T3 than those present in humans and lack the need to increase T3. However, in conditions of hypothyroidism in both rats and humans, DIO2 can be induced to increase conversion of T4 to T3 (Wagner et al., 2003). DIO3 is expressed in brain, liver, intestine, placenta and pregnant uterus with much higher activity in fetal than in adult tissues. It catalyzes the inactivation of T3 and T4 (Gereben et al., 2008).

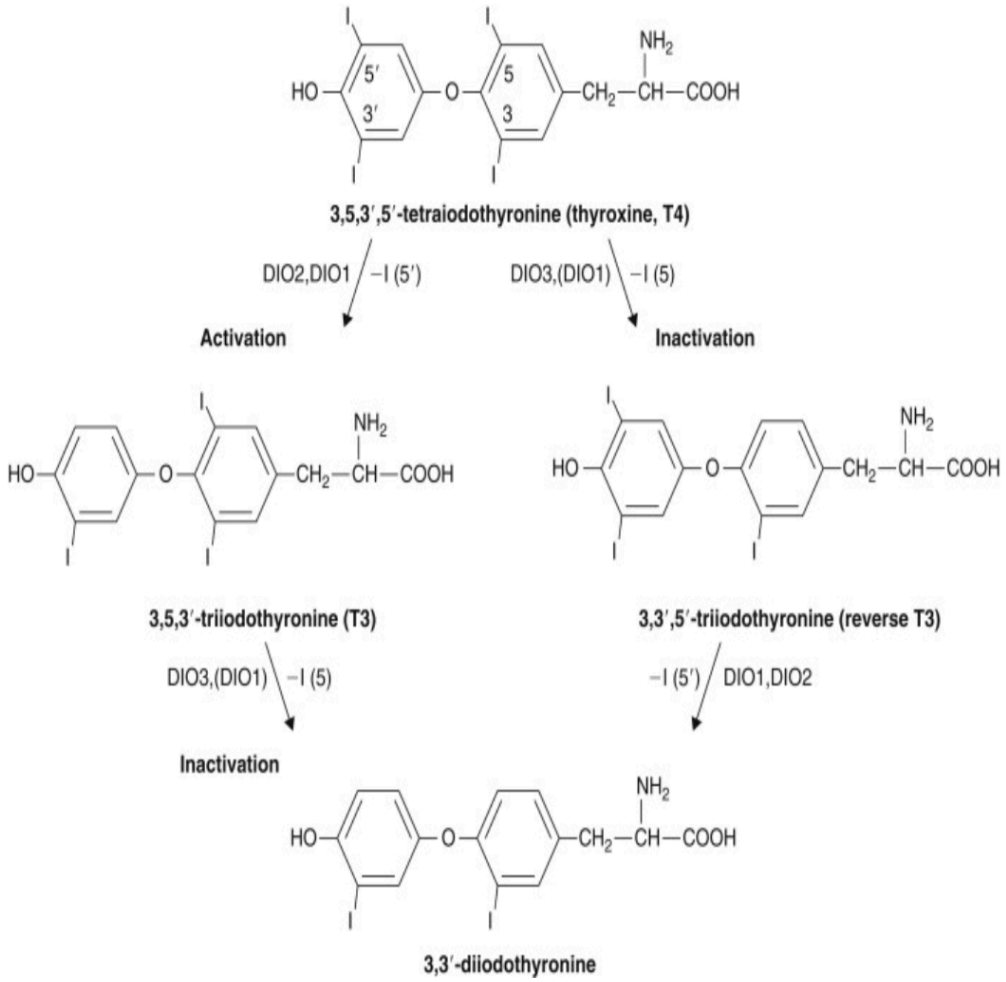

Figure 5. Iodothyronines produced by the thyroid gland and by metabolic deiodination in peripheral tissues. Type 2 deiodinase converts T4 into the major active ligand, T3. Type 3 deiodinase mediates inner-ring deiodination of T4 or T3 to form the largely inactive metabolites rT3 and 3,3'-T2, respectively (Forrest et al., 2017; Guo et al., 2009)

The best understood cellular actions of TH are carried out via receptors found constitutively in the cell nucleus. Ligand-activated receptors directly control the transcription of specific sets of target genes that carry out cell and developmental stage specific responses. There are two well conserved TH receptor gene loci in all vertebrates, designated TRα and TRβ. **Thyroid hormone receptors (TRs)** are the point in the response pathway closest to the endpoints that result in altered cellular responses: the proper transcriptional control of species sets of genes. The relatively snug fit of T3 in the TR ligand binding domain only translates to those chemicals with a structure closely resembling that of the hormone itself and which specifically interact with the TR ligand binding domains with reasonable affinities. An example of those chemicals is TBBPA or OH-PCBs (Cheek et al., 1999; Hofmann et al., 2009). A functional consequence of agonists binding to the TR ligand binding domain is co-activators recruitment, and antagonists that have been described for TRs act at least in part via disruption of this interaction (Murk et al., 2013).

1.2.5 Species differences

Although the basic physiology of thyroid hormone pathways is conserved over the different species, there are a number of important differences in transport, excretion and metabolism

of thyroid hormones between rats/mouse and humans as already mentioned in the different subchapters. Those differences hinder the transfer from results obtained in rat studies to the human situation. In the following table some main differences between those species have been summarized.

Table 2: Comparison of thyroid function and control between humans, rats and mice from data obtained from in vivo (rat &mouse) and clinical studies (human). Adopted from (Bartsch et al., 2018; Choksi et al., 2003; Colnot & Dekant, 2017; Jahnke et al., 2004; Lewandowski et al., 2004)

Parameter	**Human**	**Rat**	**Mouse**
Half-life of T4	5-9 days	0.5-1 day	0.5-0.75 days
Half-life of T3	1 day	0.25 days	0.45 days
High affinity TBG	Present	Absent	Absent
Primary serum binding protein for T4	TBG	Albumin (TTR)	Albumin (TTR)
Primary serum binding protein for T3	TBG	Albumin	Albumin
Serum TSH levels	1	5-10 x higher	Unknown
T4 production rate	1	10 x higher	Unknown
Sex difference in serum TSH level	Males = females	Males>females	Males>females
Sex ratio for thyroid cancer	Females>males	Males>females	Males>females
Effect of chronic TSH stimulation	Goitre	Cancer	Cancer
Amount of T4 supplementation required in absence of functioning thyroid	2.2 mg/kg bw/day	20 mg/kg bw/day	Unknown
Development of foetal HPT	TSH/T3 by week 20 of gestation	TH & TSH by day 17 gestation	Unknown
Morphology of the follicular epithelium	Low epithelium	Tall cuboidal epithelium	Tall cuboidal epithelium
Morphology of the follicles	Large, lots of colloid	Small, little colloid	Small, little colloid
T3 glucuronidation	Absent	Major route	Unknown
Type 2 deiodinase expression in thyroid	High	Very low/absent	Very low/absent
% of T4 eliminated in bile	10-15%	~50%	Unknown

Also, in terms of development during pre-, peri- and postnatal periods there are differences between species, specifically in the timing of neocorticogenesis between rat and human with respect to stages of pregnancy. Neocorticogenesis is the growth and development of the neocortex, which is involved in higher- order brain functions such as sensory perception, cognition, generation of motor commands, etc.

In humans, neocortical development occurs between week 6 and week 24 of gestation, although most cortical cell migration occurs between week 8 and week 24 and mostly before

the onset of fetal thyroid hormone secretion, which occurs at midgestation. In the rat neocorticogenesis begins comparatively later at about embryonic day 13 and is complete around the timepoint of birth. Most of the process occurs between gestational day (GD) 14 and 19, again mainly before onset of fetal thyroid function (aprox. at GD18). Moreover, the postnatal development and maturation of the CNS takes comparatively longer in humans than in rats. Despite the differences in timing of neurodevelopmental events between these species, similarities might be established when onset of fetal thyroid gland secretion is taken as the reference point (Berbel et al., 2007).

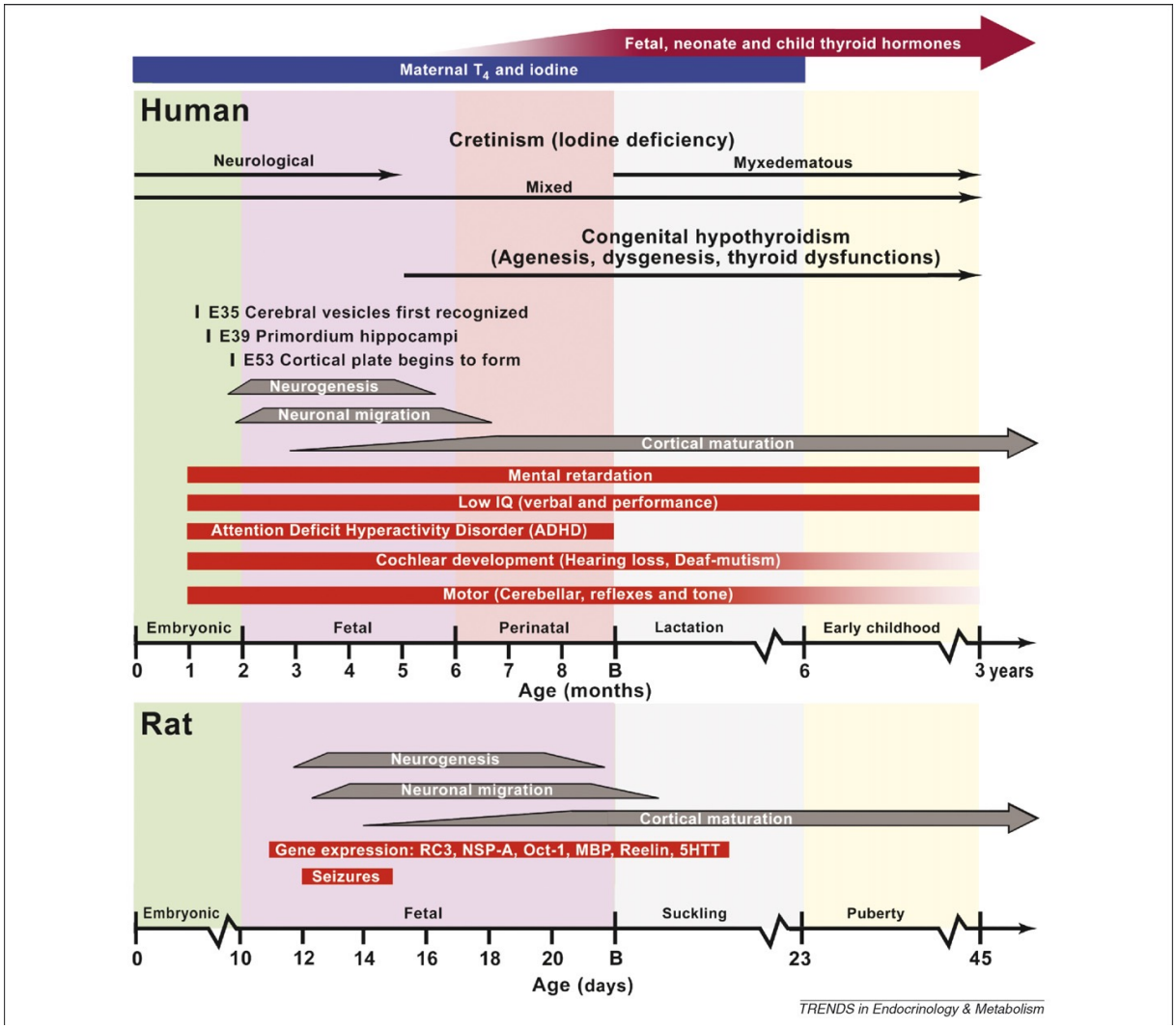

Figure 6. Main neurodevelopmental events and neurological alterations associated with iodine deficiency during fetal and neonatal life. Time scales and the respective developmental periods are indicated for humans (upper) and rats (lower) panels. The developmental periods are highlighted with different background colors. The period in which the child's thyroid hormone is secreted is indicated by the upper brown arrow. The gradation indicates the period in which the production of fetal thyroid hormones increases to reach neonate values. The period in which both T4 and iodine are transferred from the mother to the fetus is indicated by a blue bar. Some major developmental events of the cerebral cortex are also indicated for both humans and rats. Thyroid hormone deficiencies and their etiologies, which are indicated in parentheses, are shown in the upper part of the human panel. Thin black arrows represent the crucial periods related to these disorders. Some neurological alterations associated with maternal/fetal T4 and iodine deficiency and crucial vulnerability periods are shown by red bars. In the lower panel (rat), red bars indicate genes that are regulated by thyroid hormones and behavioral alterations associated with maternal and/or fetal T4 and iodine deficiency. For cochlear and motor disorders, heavy gradation indicates the period in which the brain is more sensitive to iodine deficiency (Berbel et al., 2007).

In humans in the case of severe iodine deficiency (when urinary iodine levels are lower than 20 mcg/L) the synthesis of TH is inadequate. Maternal hypothyroxinemia results in children born with irreversible CNS damage with abnormalities including mental retardation, deficits in hearing and speech, motor alterations etc. The severity of the neurological damage depends both on the developmental period when the iodine deficiency occurs and on severity of the deficiency. The earlier the iodine deficiency occurs and the greater the severity, the more devastating the neurological damage, and the first half of pregnancy is an especially sensitive period for the occurrence of irreversible CNS damage. Also, in rat and mouse studies that focus on the role of maternal thyroid hormones in the development of the cerebral cortex it has been shown that the maintenance of normal levels of maternal thyroid hormones during early pregnancy, especially that of circulating T4, is very important for the normal neurodevelopment of the progeny (Berbel et al., 2007; Gilbert et al., 2020). Brain structural changes have been shown to occur on adult cretins which were tested with non-invasive methods like MRI (De Escobar et al., 2007). However, it is still not clear if those changes are due to maternal hypothyroidism or also hypothyroidism of the children themselves since both mothers and children are living in the same iodine deficient areas. Moreover, a change in free T4 concentration during pregnancy was associated with lower child IQ and lower grey matter and cortex volume in a population-based prospective cohort study (Korevaar et al., 2016).

Despite the species differences, rodent models of developmental hypothyroidism have provided essential information about the hormonal control of brain development. Most of these studies are able to model the human condition of cretinism. To achieve that a severe TH reduction is induced in rats. Besides methods like thyroidectomy manipulation of TH levels is done through administration of chemicals like PTU, which inhibits TH synthesis and has shown that offspring aged to adulthood exhibit deficits in brain synaptic physiology. Permanent alterations in hippocampal synaptic transmission resulting from transient developmental TH insufficiencies are indicative of impaired neural circuit function (Gilbert et al., 2020). Most TDCs have the ability to reduce serum T4 in rats, nevertheless the direct implications to impaired neurodevelopment remain unclear. It is possible that TDCs with a mode of action similar to that of PTU may mimic the *in vivo* effects, however, additional information is critical to determine how an exposure to those chemicals may affect brain development. Unfortunately, the *in vivo* mechanisms of action for many chemicals are unknown and therefore, it is difficult to predict how these xenobiotics may or may not affect serum THs, brain THs and brain action. In this thesis a trial for clarification of the AOP of some TDCs is made with further goal to explore if PTU is maybe an exception to other TDCs in terms of its potential to impair brain function.

2 Methodology

2.1 Literature research

For understanding the mechanisms underlying TDCs leading to neurodevelopmental changes, an evaluation of *in vivo* mammalian regulatory toxicity studies was done in order to create a database with relevant information as well as to assess which KEs in thyroid related AOPs are experimentally addressed in studies with rodents.

Based on the EFSA Guidance for submission of scientific peer-reviewed open literature (EFSA, 2011) the relevance criteria should not be too restrictive, in order to avoid missing relevant studies. For toxicological and metabolism studies an example for the criteria of relevance is given:

1. Well defined test material (including its purity and impurity profile)

2. Relevant test species (to the mammalian toxicological assessment - preferred species are rodents - rats and mice, the dog is the preferred non-rodent species)

3. Number of animals per group sufficient to establish a statistical significance

4. Several dose levels tested (at least 3), preferably including a negative control, to establish a dose-response

5. Relevant route of administration in terms of risk assessment (oral, dermal or by inhalation)

6. Description of the observations, examinations, analysis performed, or necropsy

The literature search for the six selected reference compounds was done using the databases of *toxcenter* and *embase*. The publications retrieved that fulfilled the inclusion criteria were further analyzed and prioritized based on the relevance of the information for the parameters of the database to be established. Those are in-life, blood/tissue and pathology/histopathology parameters.

The following search query was used: CAS-No. and thyroid# and (rat or rats or rattus or mouse or mice or mus or dog# or beagle).

With this query a search was done for the substances carbamazepine (CAS-No: 298-46-4), PTU (CAS-No: 51-52-5), ammonium-, potassium-, sodium perchlorate (CAS-No: 7790-98-9, 7778-74-7, 7601-89-0) iopanoic acid (CAS-No: 96-83-3) and TBBPA (CAS-No: 79-74-7) and gave 1711 hits in toxcenter and 1187 hits in embase on 11th of September 2019.

The same query was used for the search of resorcinol (CAS-No: 108-46-3) and gave 24 hits in *toxcenter* and 12 in *embase* on 8th of December 2020.

After screening of the identified records, the articles assessed for eligibility were 9 for carbamazepine, 9 for iopanoic acid, 33 for perchlorate, 43 for PTU, 16 for TBBPA, 11 for resorcinol. To those a few relevant publications were added through non-systematic snowballing so that the final number for publications considered was: 13 for carbamazepine, 9 for iopanoic acid, 35 for perchlorate, 53 for PTU, 18 for TBBPA, 11 for resorcinol.

Publications between 1950 to 2019 were regarded. Focus was set on *in vivo* experiments on rodents with thyroid, brain and liver overall parameters investigated, looking at the effects of the compounds on euthyroid animals. *In vitro* studies, mathematical models and kinetic studies were excluded. Articles were pre-selected based upon an evaluation of title and abstract. Considering those factors, the numbers of eligible publications used for this investigation were: 9 for carbamazepine, 5 for iopanoic acid, 11 for sodium perchlorate, 39 for PTU, 7 for TBBA, and 7 for resorcinol.

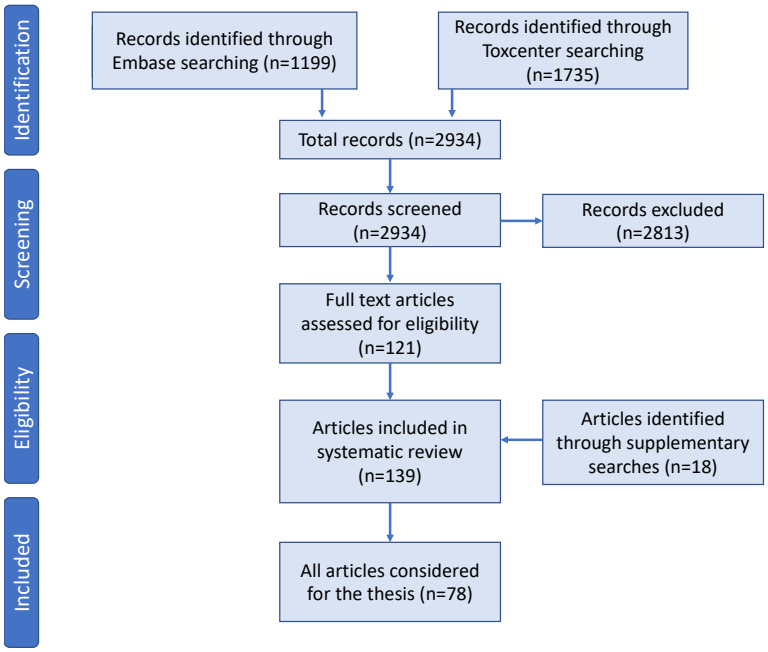

Figure 7: Flowchart of the study selection for the combination of all substances evaluated in this work. 1199 records were identified through the Embase database (12 for resorcinol and 1187 for the rest of the substances) and 1735 records were identified through the search in the toxcenter database (24 for resorcinol and 1711 for the rest of the substances) giving a total record of 2934 publications. A screening of those excluded 2813 studies. 121 studies were left to be assessed for eligibility to which 18 more publications were added through supplementary searches of random snowballing. From the total of 139 studies 78 were eligible and were used for an evaluation in this thesis.

2.2 Adverse Outcome Pathway

One of the new methodologies of risk assessment is the 'bottom up' approach in place of the previous 'top down' approach, which means instead of starting from the observed toxicity in an organism and trying to capture the effects on the organs/tissues, then cells and finally identifying the mode of action of a chemical it is done the other way around. With 'bottom up' the investigation starts from the molecular initiating events (MIE) to end up in the toxicity of the organism or the population. In that, Adverse Outcome Pathways (AOPs) play an important role but can likewise be used to elucidate MIEs from observed adverse outcomes and key events (KEs).

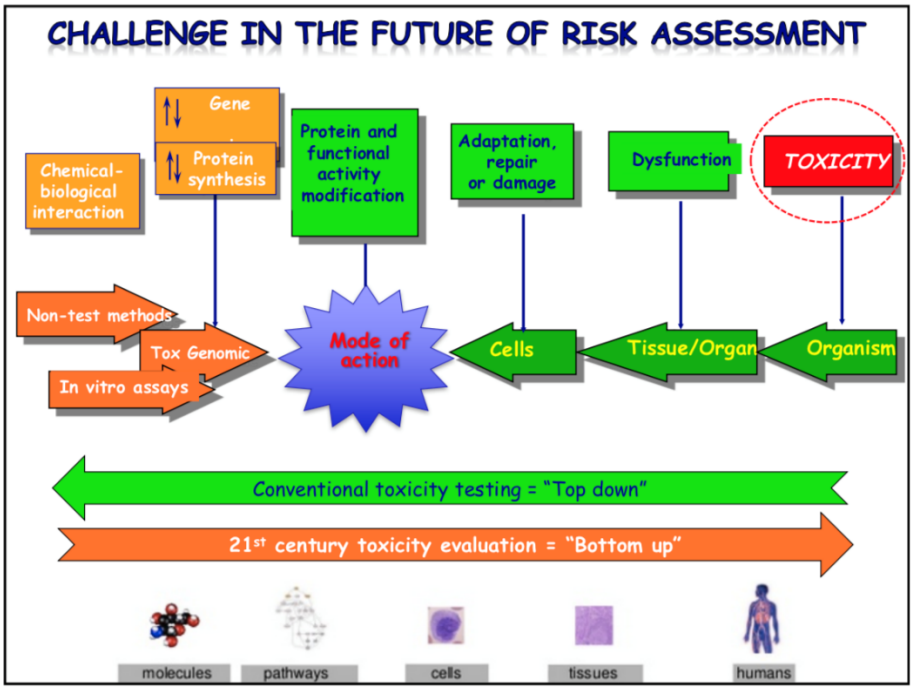

Figure 8: Bottom up vs top down approach in toxicology for the empowerment of alternative methods. Non-test methods (in silico), in vitro and toxicogenomic methods for the identification of the mode of action of chemicals in contrast to the conventional toxicity testing starting from the whole organism (Corsini lecture, university of Milan, 2019).

AOPs are conceptual frameworks that link key events (KEs) resulting from chemical or material exposure to adverse health or environmental impacts (Adverse Outcomes; AOs) important for evaluating safety. AOPs are designed as frameworks to organize toxicological information and offer a systemic and mechanistic approach to develop, assess, use, and interpret alternative testing strategies. They are expected to reduce reliance on animal testing and are anticipated to be useful in risk assessment by linking alternative testing to health and environmental effects of emerging substances in a more systemic way (Ankley et al., 2010; OECD, 2020). AOPs represent a sequence of KEs between a MIE and an AO and span many levels of biological organization molecular, cellular, tissue, organ, organism, and population. Those KEs must be measurable changes in a biological system with causal relationship between pairs of KEs. Those units can be shared between multiple AOPs. Although a biologic response to a stressor is not simplistic in a way that there might be different pathways leading to an outcome, an AOP does not try to capture all those possible ways. It defines a single sequence of biological events leading from a single initiating event to a single AO (AOP-wiki). The information for the relationships among levels of biological organization may derive from *in vitro*, *in vivo* or computational systems. AOPs provide a useful structure where existing knowledge can be arranged and from which key uncertainties and research priorities can be identified to improve predictive approaches (Ankley et al., 2010).

It is important to mention that AOPs are by definition not chemical specific. The MIE, which represents the first step in a directed cascade of dependent biological processes (KEs), is defined as a specific type of interaction of a toxicant with a biological target (e.g. receptor, enzyme). An implicit assumption underlying the AOP framework is that any chemical or stressor that triggers the MIE has the potential to elicit the chain of downstream KEs

represented in the AOP. Therefore, if a chemical has a biological activity at an MIE based on a direct measurement (eg. via *in vitro* screening) or based on its chemical structure and properties there is some confidence in predicting the types of downstream responses it may elicit (Villeneuve et al., 2014).

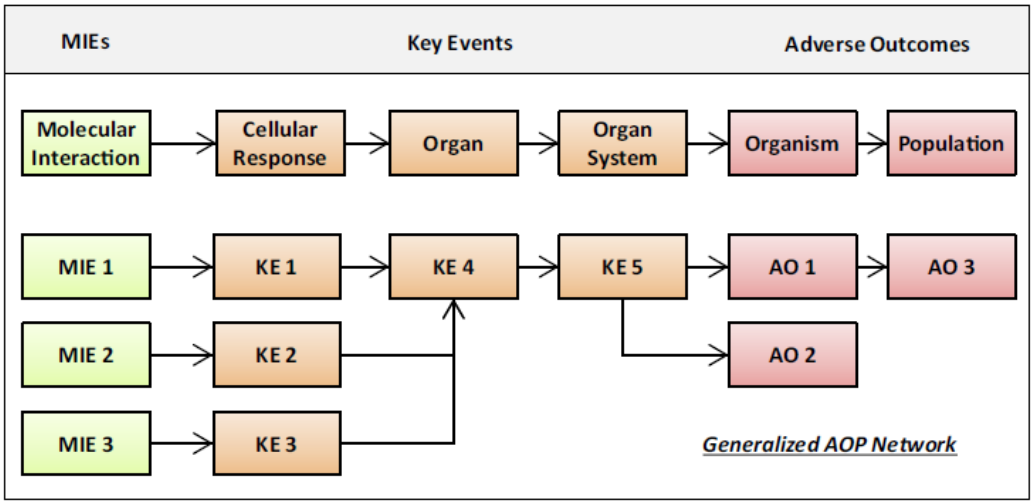

Figure 9: An AOP begins with a MIE and terminates with an AO that is linked by a series of intermediate KEs at increasing levels of biological organization (Noyes et al., 2019).

Since thyroid disruption is one of the pathways connected to the more general category of endocrine disruption following the endocrine criteria (2100/2017 and 2018/605) and the ECHA/EFSA Guidance 2018, the OECD is aiming to update the Guidance Documents for endocrine disruption with thyroid related endpoints like it has been done with the OECD TG 408 and 414 (Noyes, 2019). Moreover, an OECD Scoping Document has been published in 2014 to identify *in vitro* or *ex vivo* assays that could provide screening level information on whether a chemical has the potential to modulate thyroid hormone signaling. This project is currently coordinated by the European Union Network of Laboratories for the Validation of Alternative Methods (EU NETVAL) (OECD, 2014). The identification of chemicals with a thyroid mechanism of action is complicated due to the lack of linkages between chemical effects detected at macromolecular levels of biological organization, endpoints, and adverse outcomes. Measuring chemical interactions with thyroid-related molecular targets can help to elucidate whether adverse outcomes are mediated through a TH signaling mechanism. The KE relationships connecting MIEs to KEs and adverse outcomes may be considered hypothesized, correlative, or causal depending on the strength of the evidence.

To show the connection between the different AOPs starting from the MIE for thyroid disrupting chemicals Noyes et al. (2019) developed an AOP network that links well known and putative chemical targets of thyroid activity to downstream adverse outcomes, which is shown in the following figure (*10*).

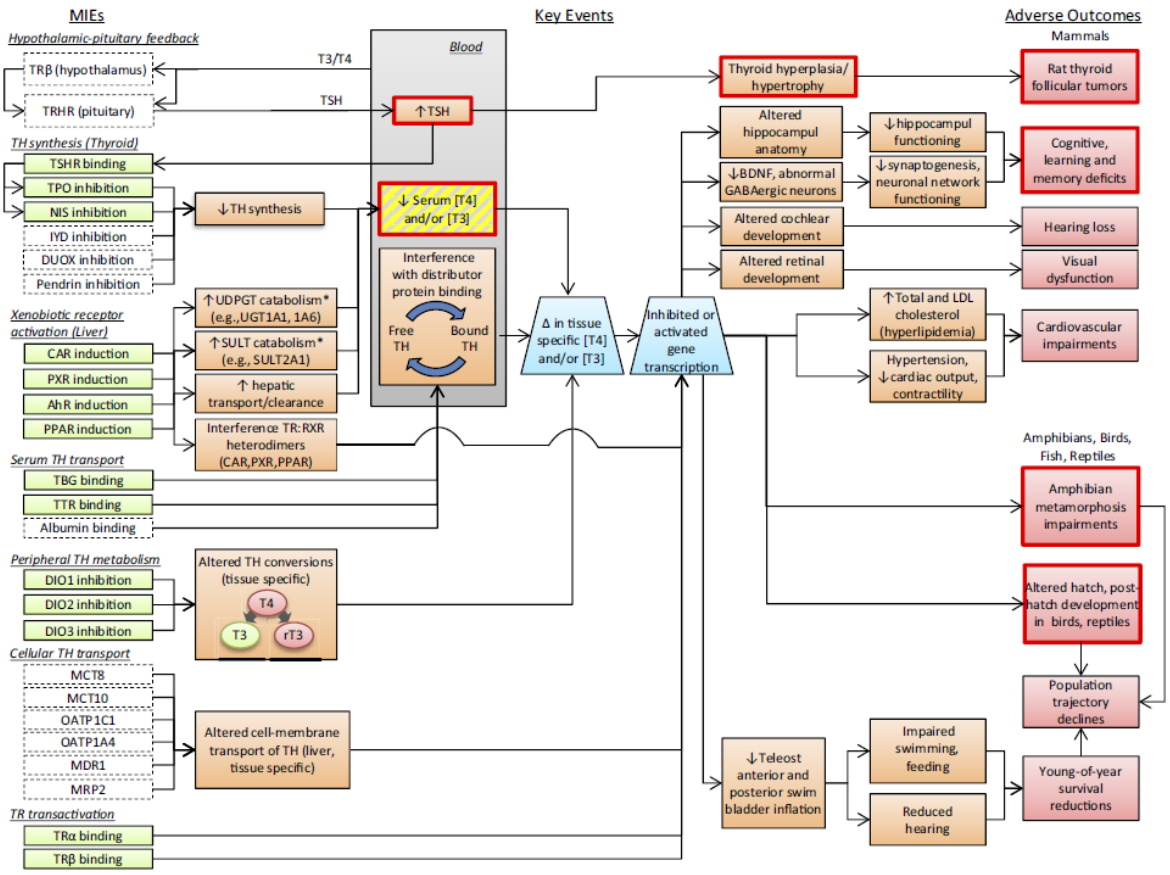

Figure 10: Adverse outcome pathway network for chemically induced thyroid activity showing the integration of multiple individual AOPs under development and proposed. Boxes with thick, red borders represent in vivo end points that are targeted by US EPA and OECD test guidelines (Noyes et al., 2019).

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.

For the investigation of the different thyroid mechanisms of action that are being considered in this work namely Thyroid Hormone Binding Protein (THBP) transthyretin, Thyroid Peroxidase (TPO), Sodium-Iodide-Symporter (NIS), Deiodinases I-III (DIO), Thyroid Hormone Receptor (TR) inhibition and Xenobiotic Receptor Activation, AOPs from the AOP wiki page were used as a basis and the observed effects from the different studies were matched to them.

THBP Transthyretin (TTR): The AOP#152 has been published in the AOP-wiki with the title '*Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity*' (https://aopwiki.org/aops/152). This AOP describes adverse neurodevelopmental effects that may result from xenobiotic interference with thyroid serum binding protein transthyretin during certain developmental windows. Binding of TTR by a xenobiotic (the MIE) leads to a displacement of T4 followed by an increased excretion of the unbound thyroid hormones by the liver. The resulting lower plasma and tissue thyroid hormone levels in early pregnancy may disrupt the normal neurodevelopment of mammals (last modified on April 16th, 2021).

Thyroid Peroxidase (TPO): A well-studied mechanism of action is the one for inhibition of TPO, which was first described and published by Zoeller and Crofton in 2005. The overall weight of evidence for this AOP#42 '*Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals*' is strong. With that pathway the implications of developmental TPO inhibition for hippocampal anatomy, function and ultimately neural function controlled by the hippocampus is discussed. Gaps in understanding of the pathway include the relationship of TH-dependent gene expression and complexities of brain development. The quantitative information of the key event relationships is limited.

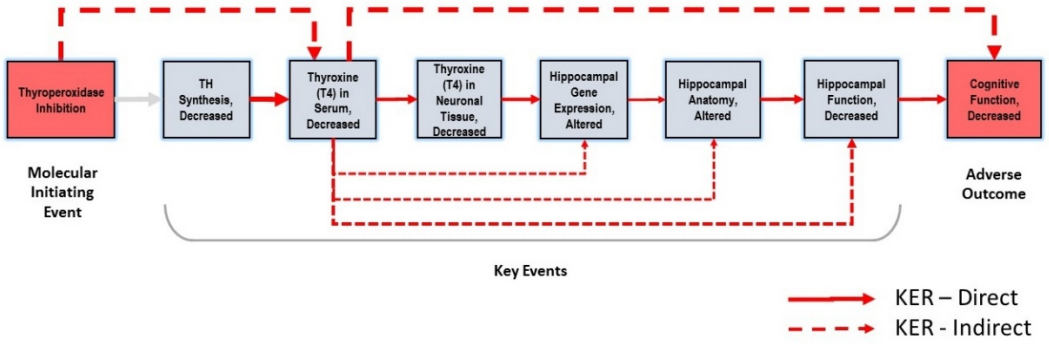

Figure 11: TPO inhibition and altered neurodevelopment (https://aopwiki.org/aops/42, last modified on August 16, 2019)

Sodium-Iodide-Symporter (NIS): *Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment* (humans) (https://aopwiki.org/aops/54). It describes the causative links between the MIE leading to the decreased levels of TH in the blood and then brain, causing memory and learning deficits in children. The overall weight of evidence for AOP#54 is strong. The function of NIS and its essentiality for TH synthesis is well known across species, however, quantitative information of KEs is limited. Three key events of this AOP (decrease of TH synthesis; T4 in serum and T4 in neuronal tissue) are common with AOP#42.

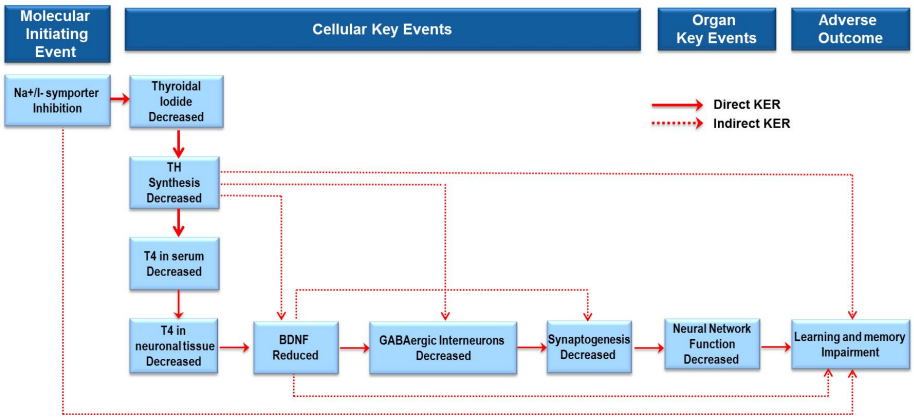

Figure 12: NIS inhibition and learning and memory impairment (https://aopwiki.org/aops/54, last modified on August 16, 2019).

Inhibition of Deiodinases 1, 2, 3: Inhibition of the peripheral as well as anterior pituitary deiodinases 1, 2 and 3 alters the TH conversion from T4 to T3 and reversed T3 (rT3) leading to change in tissue specific T4 and T3 concentrations, altering the gene expression. That can lead to histopathologic alteration in the hippocampus followed by changed hippocampal function and synaptogenesis that could lead to cognitive, learning and memory deficits as an adverse outcome (Noyes et al., 2019).

Xenobiotic Receptor Activation: The AOP for *'Upregulation of thyroid hormone catabolism via activation of hepatic nuclear receptors, and subsequent adverse neurodevelopmental outcomes in mammals'* (https://aopwiki.org/aops/8) is an update of the WHO/IPCS MOA developed in 2005 by Crofton and Zoeller. More specifically through activation of xenobiotic nuclear receptors an increase in phase II catabolism and hepatic transport occurs, which might decrease serum T3 and T4 and subsequently tissue T3 and T4 concentrations. This can lead to altered neurodevelopment and cochlear damage followed by neurological dysfunction and hearing loss respectively (last modified on June 17th ,2020).

Thyroid Hormone Receptor inhibition: Binding to the thyroid hormone receptor TRα or TRβ can inhibit or activate the gene transcription, which then leads to either an altered hippocampal anatomy and subsequent decreased hippocampal functioning or a decrease in brain derived neurotrophic factor (BDNF)/ formation of abnormal GABAergic neurons and a subsequent decrease in synaptogenesis, and neuronal network functioning. Both of those ways can lead to the adverse outcomes of cognitive, learning and memory deficits (Noyes et al., 2019).

2.3 Chemical substances

In this work the substances tetrabromobisphenol-A (TBBPA), 6-propylthiouracil (PTU), perchlorate, iopanoic acid, carbamazepine and resorcinol were investigated.

TBBPA is a flame retardant widely detected in the environment and is known to be transferred from dams to fetuses and offspring through the placenta and milk respectively (Nakamura et al., 2007). Based on *in vitro* studies TBBPA has shown to inhibit all three deiodinases (DIO1, DIO2, DIO3) (Olker et al., 2019). Moreover, there are indications for an activity of TPO inhibition but with a low selectivity score (Paul Friedman et al., 2016) as well as for TR antagonist activity (Paul-Friedman et al., 2019). TBBPA is also a potent competitor of TTR (Meerts et al., 2000). Furthermore, it has shown to have a liver enzyme inducing potential (Choi et al., 2011; Cope et al., 2015).

PTU is a popularly used antithyroid drug (ATD) for Grave's hyperthyroidism. ATDs transmitted through the placenta or milk have been shown to expose the fetuses/neonates to a risk of hypothyroidism. The disruption of perinatal thyroid function causes disorders in many networks, including the central nervous system and the immune system (Nakamura et al., 2007). *In vitro* studies show that PTU has a full dose response for DIOI (Olker et al., 2019) and is an active and highly selective TPO inhibitor (Paul Friedman et al., 2016).

Ammonium perchlorate (AP) is used in a solid propellant for rockets, missiles and fireworks. Due to its limited shelf life, superannuated AP is replaced frequently with a fresh supply, resulting since the 1950s, in the disposal of large volumes of AP. It is quite soluble in water and the resultant toxic perchlorate anion is exceedingly mobile in aqueous systems. It is

resistant to reaction with other available constituent in aqueous systems and can persist for many decades under typical groundwater and surface water conditions. Perchlorate is known to be a NIS inhibitor (Wang et al., 2018), as it has a similar ionic size with iodide and can be transported into thyroid follicular cells (Thuett et al., 2002).

Resorcinol is used in large quantities in the manufacture of tires and rubber goods, in the production of resins, and in dyeing applications. Much smaller quantities are incorporated into pharmaceutical creams to treat acne and into cosmetic products, notably hair dyes. Resorcinol was first introduced into medicinal use in the 19th century to treat, amongst other conditions, ulcerating skin lesions (Welsch et al., 2008). Based on *in vitro* studies resorcinol seems to be an active TPO inhibitor (Paul Friedman et al., 2016).

Iopanoic acid is a radiographic contrast agent that is known to inhibit T4 to T3 conversion in man and experimental animals. (St Germain, 1988). Also in *in vitro* assays it has shown to inhibit the deiodinases (Olker et al., 2019).

Carbamazepine is a major drug in treatment of epilepsy that shows less pronounced toxicity in comparison to other antiepileptic drugs. However, the treatment must be discontinued in 5% of the cases due to serious side effects (Kubová & Maresš, 1993). Based on in *vitro ToxCast* tests carbamazepine has shown to be activating the xenobiotic receptor PXR but not the receptor CAR (Wang et al., 2012).

2.4 Reliability of the data

In this thesis all the data considered are from already existing studies, mainly from publications. Therefore, it was important to evaluate if these data are complete and valid. In other words, considering their quality and adequacy. More specifically, the data have to be reliable but also the test methods should be validated to prove their relevance and reproducibility. That is important to also being able to compare results in case of conflicting studies. The more details on methodology, test procedures, and analytics are documented the easier is the evaluation of their reliability.

Reliability refers to the extent to which a study is free from bias and its findings reflect true facts. Some principles that may be considered when assessing reliability of the studies are statistical power, verification of measurement methods and data, control of experimental variables that could affect measurements, universality of the effects in validated test systems using relevant animal strains and appropriate routes of exposure, and biological plausibility of results (EFSA, 2011).

In this study the categorization of the data for their reliability was done based on the Klimisch code (Klimisch et al., 1997):

1. Reliable

These are studies, which were carried out or generated according to generally valid and/or internationally accepted testing guidelines, preferably performed according to good laboratory practice (GLP).

It must be emphasized that compliance with GLP standards should not be considered as a guarantee for reliability. Study reliability must be judged solely on the basis of the accuracy and reproducibility of the facts reported. (EFSA, 2011)

2. Reliable with restrictions

These are studies, in which the test parameters documented do not totally comply with the specific testing guideline but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.

The following information should be given:

- Data/information on the test animals (species, strain, sex, age)
- Purity/composition/origin of the test substances
- Number of animals evaluated
- Scope of the investigations per animal (clinical chemistry, hematology, organ weights etc. and description of the methods
- Description of the changes/lesions observed
- Control group or histological control data of the laboratory
- Description of the test conditions
- Description of the route and doses of administration
- Dose/concentration relationship if possible.

3. Not reliable

These studies include data with interferences between the measuring system and the test substance or studies which were carried out with a method that is not scientifically accepted or the documentation of the experiments and the results is not sufficient for an assessment and is not convincing for an expert judgment.

For example, some points that were always taken into consideration in this thesis is if a control group was used, if enough doses were given to the animals and if enough animals were used in order to have a result with statistical significance. If those points were not covered the study was categorized as non-reliable.

4. Not assignable

These are studies, which do not give sufficient experimental details, and which are only listed in short abstracts or secondary literature (books, reviews, etc.)

This procedure of categorization is not intending to automatically exclude information with low reliability from further consideration. The use of this reliability assignment is for being able to use the weight of evidence approach in cases where the information from a single piece of evidence alone is not sufficient to fulfil an information requirement (for example due to deficiencies in studies) or when individual studies provide conflicting conclusions.

The weight given to the available evidence depends on quality of the data, consistency of results, nature and severity of effects, and relevance of the information (ECHA).

3 Results

The literature research was done on rodent studies with thyroid related parameters for the six reference compounds carbamazepine, TBBPA, perchlorate, iopanoic acid, resorcinol and PTU. Results that were registered after the administration of those substances to euthyroid animals are described below:

3.1 Carbamazepine

In the literature search after considering the eligibility and relevance of data, 13 studies were evaluated for carbamazepine. Dependent on the study the focus was set on effects of that compound on the thyroid but also the brain and liver. The administration of carbamazepine on Sprague Dawley and Wistar rats through the diet or intraperitoneally (i.p.) has shown to decrease the concentration of T3 and/or T4 in serum in the five studies where this endpoint was assessed (Ahmed & El-Gareib, 2017; Baumgartner et al., 1994; Baumgartner et al., 1997; Joffe et al., 1988; Villa & Alexander, 1987) starting from a dose of 25 mg/kg bw/d (Ahmed & El-Gareib, 2017). An increase in TSH was seen less often, more specifically it was assessed in four studies and showed a change only in one of them (Ahmed & El-Gareib, 2017; Oliva et al., 2021). In the studies used for this investigation there was mostly no assessment of thyroid histopathology performed. In Villa et al., 1987 after 42 days of increasing carbamazepine dosage in the diet from 40 mg/kg bw/d to a final concentration of 800 mg/kg bw/d for the last 14 days, a 25% increase in thyroid gland weight was observed but no morphological differences were seen. On brain morphology there were inconclusive results in offspring after i.p. administration (Ahmed & El-Gareib, 2017; Forcelli et al., 2011; Manent et al., 2007). More specifically, in Manent et al. (2007) with a treatment of 20 mg/kg bw/d in GD14 to GD19, there were no effects seen in terms of hippocampal and cortical dysplasia in offspring PND0 and PND30. However, the number of animals in this study is not given and therefore the evaluation of this negative result should be regarded with caution. Moreover, in Forcelli et al. (2011) there was no cell death observed in any brain region. Even so, this was a single dose (100mg/kg bw/d) on PND7 with the examination taking place on the same day. In the study of Ahmed & El-Gareib (2017) there were brain morphological changes mentioned that were however of very poor histopathological quality and could not be considered as reliable for this thesis. There were no behavioral studies done.

Based on *in vitro* screening carbamazepine has shown to activate hepatic nuclear receptors (PXR) (Wang et al., 2012). According to the literature evaluated and taking the AOP#8 on '*Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals'* as a basis the following scheme was created.

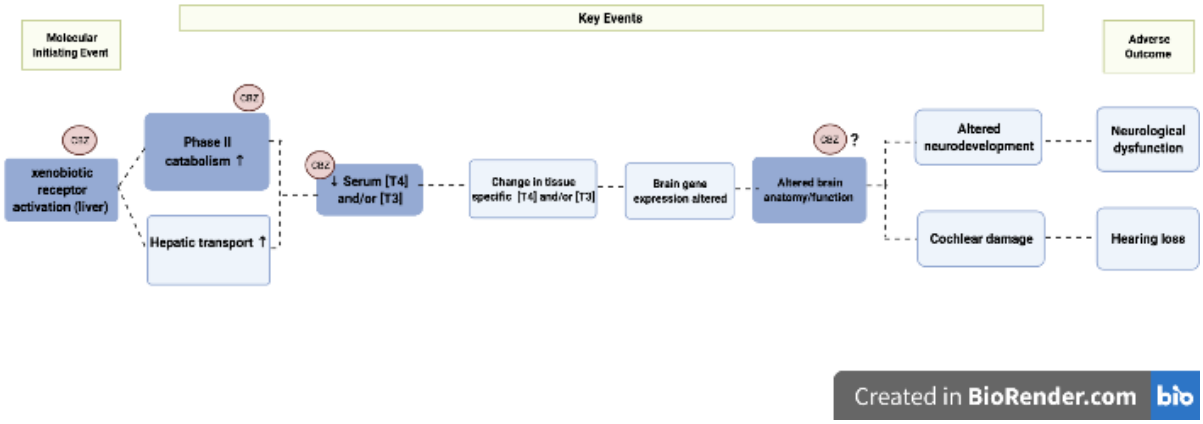

Figure 13: *AOP#8 on 'Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals' with observed carbamazepine related KEs. The dark blue boxes represent the endpoints that have been assessed in those studies.*

3.2 Tetrabromobisphenol-A (TBBPA)

After the literature was assorted 8 studies were evaluated for TBBPA. With an administration of that chemical to Sprague-Dawley or Wistar rats through food or gavage, a T4/T3 decrease in serum was observed in five out of six studies that assessed this endpoint (Choi et al., 2011; Cope et al., 2015; Nakamura et al., 2007; Saegusa et al., 2009; Van der Ven et al., 2008). It is worth mentioning that in the one study that the TH concentration in serum did not change the dose was relatively low (5 mg/kg bw/d). However, that experiment caused a TSH increase (Meerts et al., 1999). That was not the case in the other three studies that assessed the concentration of TSH in serum.

Five studies assessed the histopathology of the thyroid gland (Choi et al., 2011; Cope et al., 2015; Imai et al., 2009; Saegusa et al., 2009; Van der Ven et al., 2008). All publications except for Imai (2009) showed a T4 or/and a T3 decrease, whereas in Imai study (2009) the TH changes were not assessed. Three out of these five publications, seem to have detected an effect on the histopathology of the thyroid. An increase in thyroid weight was observed in two studies (Imai et al., 2009; Saegusa et al., 2009), although in the second study the tendency was not dose-dependent. In contrary, one study showed a thyroid weight decrease (Choi et al., 2011). Moreover, a tendency for follicular cell hypertrophy was observed but only for a dose starting from 50 mg/kg bw/d (Saegusa et al., 2009). Furthermore, follicular adenomas and carcinomas were registered although without an assessed TH change (Imai et al., 2009). However, in the studies Cope et al., 2015 and Van der Vens et al., 2015 with doses up to 1000 and 3000 mg/kg bw/d respectively, histopathological effects in the thyroid were neither seen in a subacute (OECD 407), in a one generation toxicity study (OECD 415) (Van der Ven et al., 2008) nor in a two generation toxicity study (OECD 416) (Cope et al., 2015).

A dose of 300 mg/kg bw/d did not show any effects on the CYP liver enzyme in a 28-days study (Germer et al., 2006). The hepatic T4 UDPGT activity was also assessed but showed no effect (Meerts et al., 1999). However there are results that describe a CAR activation and Cyp2B1 induction (Choi et al., 2011) , which account for the 'Xenobiotic Receptor Activation' as an MIE.

Looking at the effects on the brain, Cope et al. (2015) observed a thinning of the brain parietal cortex in F2 generation in PND11 after administration of 1000 mg/kg bw/d. However, that doesn't seem to be a relevant effect, since PND11 is not an optimal timepoint to conduct brain pathology. Indeed, this change was not associated with histological changes in the parietal cortex and was not associated with significant changes in any offspring motor activity, auditory or emotionality tests. Also, the effect of thinning of parietal cortex was not observed at later stages of development or in F1 generation. Moreover, in Saegusa et al. (2009), in which the tests were done on PND20, there were no effects seen on the brain, although this study is not completely comparable, since the dose is much lower (max 500 mg/kg bw/d). There was no selective accumulation of TBBPA in brain and no effect on type II brain deiodinases with a dose of 5 mg/kg bw/d (Meerts et al., 1999). Moreover, there was no change in neuronal migration and oligodendroglial development with a dose up to 500 mg/kg bw/d in GD10 to PND20 (Saegusa et al., 2009). There were no data regarding brain function and behavior.

Based on *in vitro* and *in vivo* data TBBPA might be responsible for several MIEs that can lead to cognitive impairment. More specifically, it is a potent competitor of TTR (Meerts et al., 2000), a TPO inhibitor (Paul Friedman et al., 2016), a DIO inhibitor (Olker et al., 2019), a TR antagonist (Paul-Friedman et al., 2019) and might also be a xenobiotic receptor activator (Choi et al., 2011; Cope et al., 2015). Therefore, an AOP network was created, which integrates the published AOPs for all these MIEs and can be used to compare those KEs to the observed events in the different evaluated TBBPA studies. The single AOPs were taken from the AOP wiki page and the publication of Noyes et al. (2019) and were combined to produce a network.

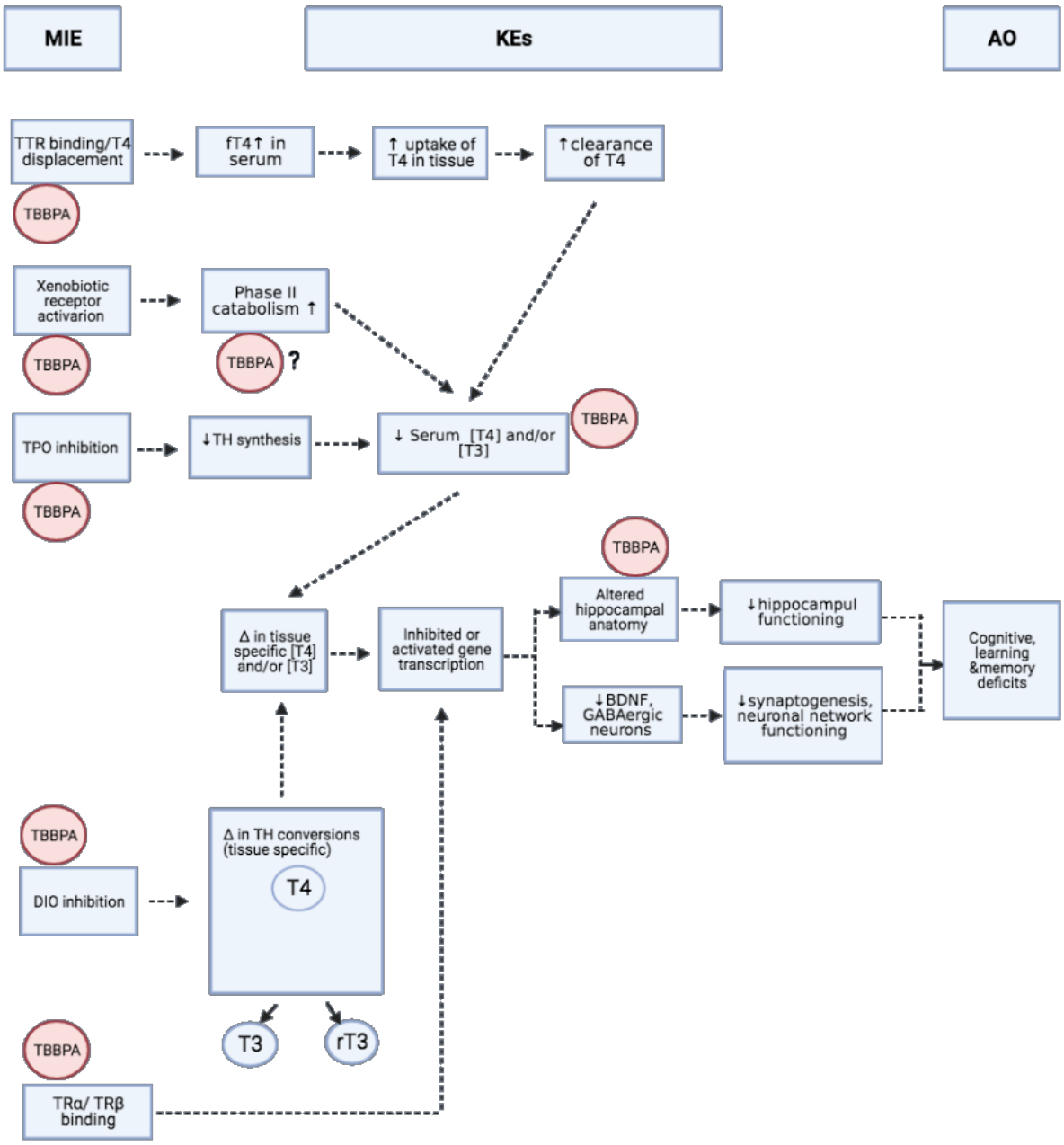

Figure 14: *AOP network for TBBPA considering the five MIEs of the substance. This includes the AOP #152 'Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity', AOP #8 'Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals', AOP #42 'Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals', AOP #300 'Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals', which is still under development and information from Figure 2 from Noyes et al. (2019) on the pathway of TR binding and DIO inhibition.*

3.3 Perchlorate

After the selection of publications 11 studies were considered for the evaluation of perchlorate administration on rodents. Different strains were used with the most frequent one being the Sprague Dawley rats. The dose was given mainly through drinking water. From the 11 evaluated studies, 9 of them assessed the TH changes in serum with 8 showing a T3/T4 decrease (Chen et al., 2015; Gilbert & Sui, 2008; HIASA et al., 1987; James-Walke et al., 2006; Serrano-Nascimento et al., 2018; Siglin et al., 2000; York et al., 2001; York et al., 2003). Eight studies assessed the TSH concentration in serum and all of them showed a TSH increase. All

those studies had also shown a T3/T4 decrease, which elucidates causality of those events. Furthermore, there is evidence for histopathological changes of the thyroid gland. Specifically, a significant increase in thyroid weight was seen (HIASA et al., 1987; Imai et al., 2009; Serrano-Nascimento et al., 2018; Siglin et al., 2000; York et al., 2001; York et al., 2003), as well as reduction in follicle size (Serrano-Nascimento et al., 2018) , follicular cell hyperplasia (Imai et al., 2009; York et al., 2001) and hypertrophy (York et al., 2001; York et al., 2003).

In the study of Serrano-Nascimento (2018), after the treatment the animals were euthanized, and the thyroid gland was excised, the RNA extracted, and the gene expression was evaluated by Real-Time PCR. The chronic perchlorate exposure (60 days) induced a massive increase of NIS expression (↑472%). It also augmented the protein content of the thyroid transcription factors of Pax8 (↑67%) and NKX2.1 (↑34%) with a dose of 3.5 mg/kg bw/d.

Moreover, an increase in hypothalamic TSH expression was shown (Serrano-Nascimento et al., 2018). In Gilbert et al. (2008), a decreased baseline synaptic transmission with 3, 30 and 100 mg/kg bw/d through the drinking water was observed. However, there were no changes in behavior, motor activity, spatial learning or fear in the treated groups.

Since perchlorate is, also based on *in vitro* methods, a NIS inhibitor a comparison of the observed effects and the KEs of AOP #54 is done.

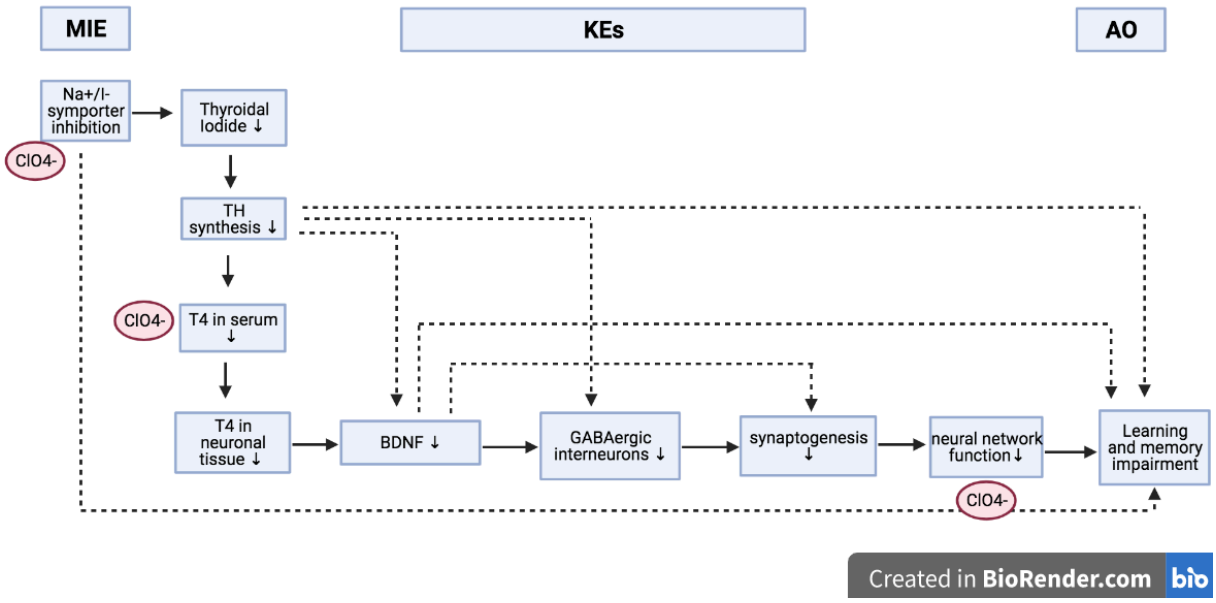

Figure 15: AOP#54 for Inhibition of NIS with integrated observed perchlorate KEs.

3.4 Iopanoic acid

After evaluation of the available studies investigating the effect of iopanoic acid on thyroid and brain, five of them were relevant for the purposes of this thesis. For the experiments of these studies rats of the stains Sprague Dawley, Wistar and F344 were used and the administration was always done intraperitoneally. In four out of five studies the change in thyroid hormone concentration in serum was assessed. All of them showed a T3 decrease and

a T4 increase with doses of 1.6, 20 and 40 mg/kg (Castro et al., 1986; Redjem et al., 1990; Weiss & Burns, 1988).

It was also shown that T4 deiodination is inhibited (Redjem et al., 1990). In Takizawa et al., 2016 an increased thyroid weight was observed with a dose of 30 mg/kg bw/d and a liver weight decrease with doses of 30 and 100 mg/kg bw/d. No brain or behavioral experiments were done for this substance.

As a basis for the comparison of KEs, the AOP for DIO inhibition from Noyes et al. (2019) was used as reference, since iopanoic acid has shown to be a DIO inhibitor *in vitro* (Olker et al., 2019) as well as in man and experimental animals (St Germain, 1988).

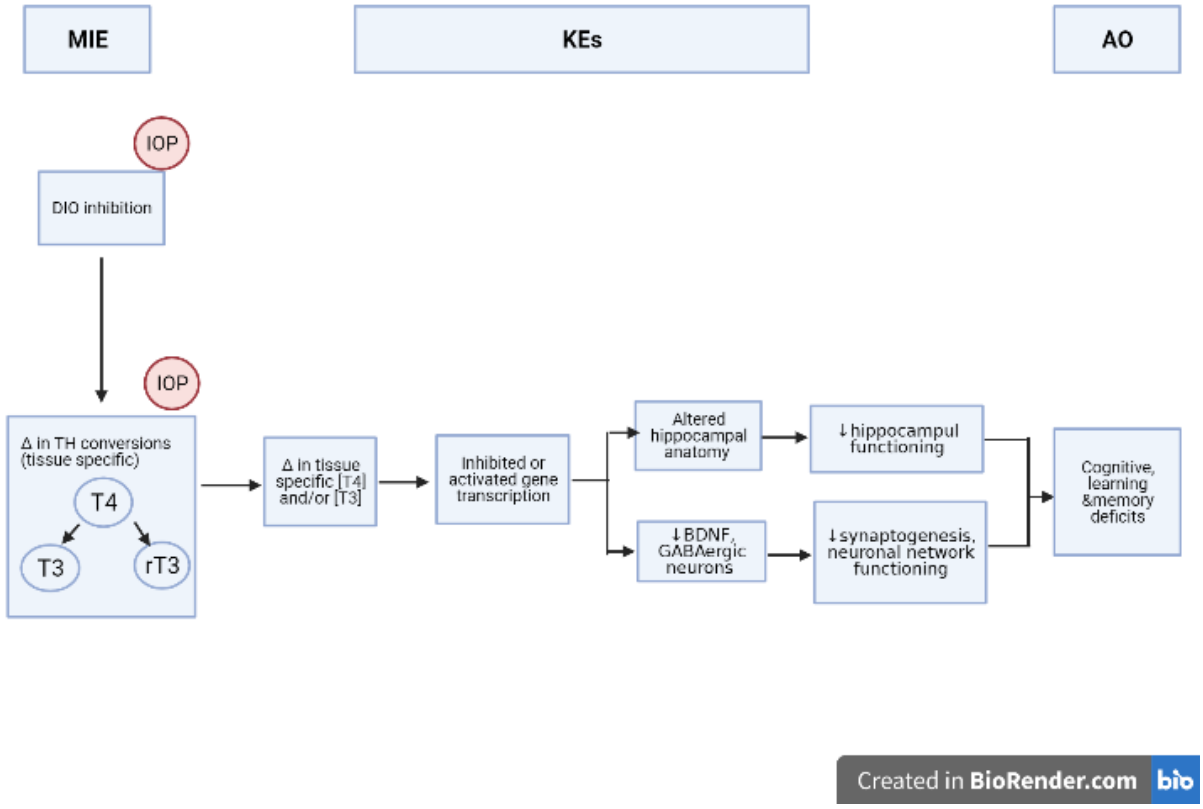

Figure 16: AOP for DIO inhibition adopted from Noyes et al., 2019 with observed perchlorate related KEs.

3.5 Resorcinol

After the selection of publications, 7 studies were considered for the evaluation of resorcinol administration on rodents. Dosing was done mainly through water and/or diet and subcutaneous administration. The TH change in serum was assessed in three studies. (Berthezene, 1979; Merker et al., 1982; Welsch et al., 2008). When the chemical was given through the drinking water with a concentration up to 300 mg/kg bw/d there was no change in TH concentration in serum (Welsch et al., 2008). Even with a subcutaneous administration with 100 mg/kg bw/d the concentration of TH in serum did not change (Merker et al., 1982). In a study with a very high dose of resorcinol (2500 mg/kg bw/d) given through the diet, a decreased T4 concentration in plasma was observed as well as an increase in thyroid weight (Berthezene, 1979). However, only the abstract of this publication was available and so its reliability is not assignable.

The ability of the thyroid gland to take up iodine was decreased with doses starting from 9.9 mg/kg bw/d up to 2000 mg/kg bw/d (Arnott & Doniach, 1952; Cooksey et al., 1985; Doniach & Fraser, 1950). In Welsch et al. (2008) the content of follicular colloid was increased with a dose of 300 mg/kg bw/d and in Seffner et al. (1994) the follicular size was increased with only 4 mg/kg bw/d of resorcinol. However, Merker et al. (1982) saw no changes in the histopathology of the thyroid gland.

There were no changes in brain histopathology with doses up to 300 mg/kg bw/d (Merker et al., 1982; Welsch et al., 2008). It is important to mention that those studies were done with adult rats so the effects of the chemical on the fetuses and pups have not been investigated.

Since resorcinol has shown to be a TPO inhibitor (Paul Friedman et al., 2016) the AOP#42 from AOP wiki was used as a basis for the comparison of the KEs studied and observed in the relevant studies.

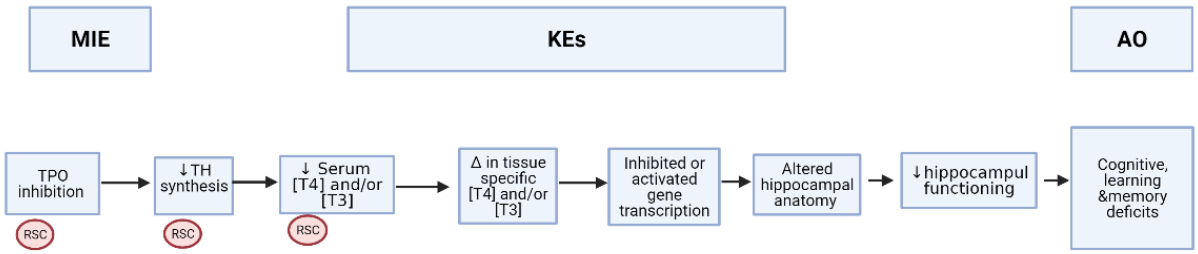

Figure 17. AOP#42 for TPO inhibition with integrated observed resorcinol related KEs.

3.6 PTU

In the 39 studies of propylthiouracil that were evaluated the chemical was given mainly through the drinking water but also by gavage. A clear decrease in TH concentration in serum was seen even with low doses of the chemical given to the animals like 0.05 mg/kg bw/d (Hassan et al., 2017), 0.1 mg/kg bw/d (Spring et al., 2016) or 0.3 mg/kg bw/d (Gilbert et al., 2017). Moreover, TSH was increased in dams and pups of several studies whenever it was assessed (Böckers et al., 1990; Chakraborty et al., 2012; Chen et al., 2018; De Sandro et al., 1991; Khaksari et al., 2009; Nambiar et al., 2014; Sawin et al., 1998; Sui & Gilbert, 2003). These effects were observed in studies where the PTU was administered to the dams before pregnancy and throughout gestation (Böckers et al., 1990; Chakraborty et al., 2012) but also when limiting the treatment in the gestation period (Hassan et al., 2017; Khaksari et al., 2009; Li et al., 2004; Mallela et al., 2014) as well as for the administration starting in the gestation period and continuing in the postnatal period, in which the offspring received a part of the dose through lactation (Axelstad et al., 2008; Gilbert & Paczkowski, 2003; Gilbert et al., 2014; Gilbert, 2011; Goodman & Gilbert, 2007; Johnstone et al., 2013; Li et al., 2004; Nakamura et al., 2007; Spring et al., 2016) (BASF_internal, 2008). Moreover, a decrease in T3 was observed in cortex and hippocampus in PND16 with a dose of 0.3 mg/kg bw/d (Spring et al., 2016).

In terms of histopathology of the thyroid gland, hypertrophy was observed with doses of 0.1 mg/kg bw/d (BASF_internal, 2008), 1 mg/kg bw/d (Chen et al., 2018) and 10 mg/kg bw/d (De Sandro et al., 1991), as well as hyperplasia (BASF_internal, 2008) (Cho et al., 2003) and an

enlarged thyroid gland (Nambiar et al., 2014). Also, a decrease in follicular size (De Sandro et al., 1991) and deformed epithelium cells was seen (Chen et al., 2018). Moreover, an increase in thyroidal weight was seen in seven out of eight studies assessed. (Axelstad et al., 2008; De Sandro et al., 1991; Gilbert et al., 2014; HIASA et al., 1987; Männistö et al., 1979; Nambiar et al., 2014). One publication showed no change in thyroid histopathology (Mallela et al., 2014) whereas others did not assess this endpoint (Behnam-Rassoli et al., 1991; Chakraborty et al., 2012; Farrokhi et al., 2014; Gilbert & Paczkowski, 2003; Hassan et al., 2017; Johnstone et al., 2013; Khairinisa et al., 2018; Khaksari et al., 2009; Li et al., 2004; Niemi et al., 1996; Smeyne & Goldowitz, 1990; Spring et al., 2016; Yang & Gordon, 1997).

In most cases of PTU administration a decreased body weight is seen (in 21 studies out of 27 that assessed this endpoint).

Looking at the histopathology of the brain a decrease in granule cell layer (GCL) area and volume (Gilbert et al., 2017) as well as cerebellar mass decrease and distorted cerebellar structure (Li et al., 2004) was observed. Also, the hippocampal volume and weight and the neocortex volume were decreased (Gilbert et al., 2017). The total brain weight was decreased as well (Niemi et al., 1996; Sawin et al., 1998). Furthermore, a subcortical band heterotopia (SBH) seems to be a common effect of PTU (Gilbert et al., 2014; Goodman & Gilbert, 2007; Spring et al., 2016). SBH is a condition in which the neurons do not migrate to their proper locations in the fetal brain. This way they cannot reach the cerebral cortex and form band-like clusters of tissue beneath that area. This abnormal brain development can cause neurological problems (NLM, 2020). Also, a decrease in long term potentiation (LTP) is often observed, which is the initiating event in memory formation (Gilbert & Paczkowski, 2003; Niemi et al., 1996; Sui & Gilbert, 2003). However, in the study of Mallela et al. (2014) there were no gross histopathological changes in brain.

A decrease in hippocampal BDNF levels in offspring (PND3 and PND7) was seen in a developmental study with a treatment of 0.4 mg/kg bw/d through the drinking water of the dams (Chakraborty et al., 2012). BDNF is important for growth and differentiation of new neurons and synapses as well as survival of existing neurons (Huang & Reichardt, 2001).

A PTU administration has shown an increase in anxiety and cognitive impairment with a dose of 17.5 mg/kg bw/d in a two-generation study with a dosing in the period GD14 to PND21 (Khairinisa et al., 2018) and a decrease in learning ability (20mg/kg bw/d) in offspring of a developmental study with a treatment from PND1 to PND60 (Farrokhi et al., 2014). Moreover, an impaired context conditioning and cue learning with a dose of 0.3 mg/kg bw/d during GD6 to PND21 (Gilbert, 2011) was seen.

As a basis for the comparison of KEs of the available studies and the published AOPs, an AOP network was created for the MIEs of TPO and DIO inhibition, which are the known modes of action for PTU (Olker et al., 2019; Paul Friedman et al., 2016).

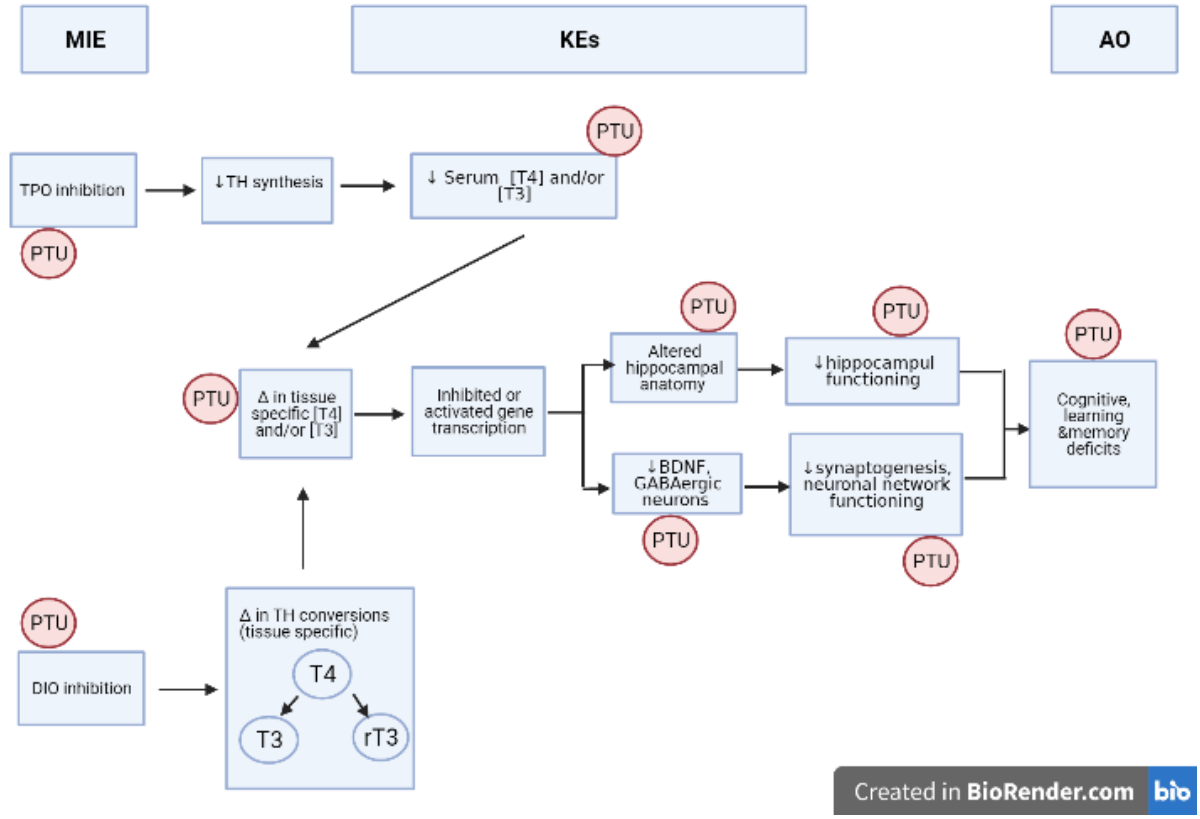

Figure 18. AOP network with the two MIEs of PTU (TPO inhibition from AOP wiki, AOP#42 and DIO inhibition based on Noyes et al., 2019) with integrated observed KEs of the substance.

4 Discussion

The Key Events (KE) and Adverse Outcomes (AOs) of the substances carbamazepine, TBBPA, PTU, perchlorate, iopanoic acid and resorcinol were studied and assorted to the already published Adverse Outcome Pathways (AOPs) of the following Mechanisms of Action (MoA) that count as Molecular Initiating Events (MIEs).

4.1 Thyroid Hormone Binding Protein, Transthyretin (TTR)

Binding of TTR by a xenobiotic and displacement of thyroid hormones may disrupt the normal neurodevelopment of mammals through a transient increase of free thyroxine (T4) levels in the circulation, followed by an increased clearance of free T4 by the liver which results in a decrease in both serum and neuronal tissues concentrations. That can lead to an alteration of hippocampal anatomy and physiology and end up to a decreased cognitive function.

Based on *in vitro* data (Meerts et al., 2000) from the substances that are evaluated in this thesis, TBBPA is the one that shows to be binding to TTR. Considering the TTR AOP that has been published on AOP wiki page with title 'Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity' the endpoints studied and observed in the rodent *in vivo* studies were compared to the single KEs of the AOP (see *table 3*). From those given KEs only a few were assessed. The concentration of TH in serum showed a decrease after TBBPA administration (KE5) (Cope et al., 2015; Nakamura et al., 2007; Saegusa et al., 2009; Van der Ven et al., 2008) and the hippocampal anatomy was altered (KE8)

(Cope et al., 2015). However, there was no change in hippocampal functioning observed when neuronal migration and oligodendroglial development was assessed. For the evaluation of the irreversible effects on neuronal migration, quantitative measurements of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region was performed in PNW11 through immunohistochemistry. Also, areas of the white matter tract with oligodendrocytes surrounding myelinated axons distributed in the cerebral cortical area were measured with immunohistochemistry to evaluate oligodendroglial development.

Free serum T4 was also assessed after administration of TBBPA (Meerts et al., 1999) but with no significant change observed. However, in this study there was in general no change in TH seen neither a binding to TTR. That might be due to the low dose of 5 mg/kg bw/d. Moreover, the used method for measuring free T4 was chemiluminescence, which doesn't give very reliable results in comparison to measurements done with dialysis (Stockigt, 2001).

There were some KEs observed in those TBBPA studies that are not described in the AOP for TTR displacement. These are the increase in TSH (however only seen in 1 out of 4 studies assessed) and some histopathological changes although for the latter there are contradictory results. More specifically, this endpoint was assessed in five studies. Two of them showed an increase in weight of the thyroid gland (Imai et al., 2009; Saegusa et al., 2009), although in the second study the tendency was not dose-dependent. In contrary, one study showed a thyroid weight decrease (Choi et al., 2011). Moreover, a tendency for follicular cell hypertrophy was observed but only when a high dose of 1000ppm (50 mg/kg bw/d) was given (Saegusa et al., 2009). Also follicular adenomas and carcinomas were registered for a study that however did not assess the changes in thyroid hormones (Imai et al., 2009). On the other hand, two investigations did not show any histopathological changes of the thyroid gland (Cope et al., 2015; Van der Ven et al., 2008).

From those data it seems like there are more investigations needed to be able to make a conclusion on whether TBBPA follows the AOP for TTR displacement based on *in vivo* data. Except for many of the KEs that haven't been assessed in those studies (eg. the concentration of T4 in neuronal tissue or the hippocampal gene expression) there are also no data available regarding brain function and behavior, which is the adverse outcome.

The causality of the events can't be proven here, since there are a lot of endpoints missing in the different studies in order to assess the connection between the KEs.

Table 3.Key Events of the AOP #152 for TTR displacement with sequence of the event, event ID and title of the event based on AOP wiki page. Moreover, it is given which KEs have been assessed in studies of the investigated chemicals of this thesis and which one of those have had an effect on rodents.

Sequence	Event ID	Title	Assessed in study of	Observed effect in study
1	958	Displacement, Serum T4 from transthyretin	-	-
2	959	Increased free serum T4	TBBPA	-
3	960	Increased uptake of T4 into tissue	-	-
4	961	Increased clearance of T4 from serum	-	-
5	281	Decreased T4 in serum	TBBPA	TBBPA
6	280	Decreased T4 in neuronal tissue	-	-
7	756	Altered hippocampal gene expression	-	-
8	757	Altered hippocampal anatomy	TBBPA	TBBPA
9	758	Altered hippocampal physiology	TBBPA	-

4.2 Thyroperoxidase (TPO) inhibition

Binding of a xenobiotic to the enzyme thyroperoxidase might inhibit its action and could lead to a decrease in TH synthesis followed by decreased T4 in serum, then T4 decrease in neuronal tissues and a subsequent alteration in hippocampal gene expression, hippocampal anatomy and function. Those events may cause a decrease in cognitive function as an adverse outcome.

From the six studied substances, two of them are known to be TPO inhibitors, based on *in vitro* enzyme activity measurement. Those are PTU and resorcinol (Motonaga et al., 2016; Paul Friedman et al., 2016). The results of the available studies on PTU and resorcinol were compared to the KEs of the well-studied AOP #42 for 'Inhibition of TPO and subsequent adverse neurodevelopmental outcomes in mammals'.

Resorcinol only showed a decrease in TH synthesis by a decrease of iodine uptake in the thyroid gland and a decrease in the concentration of TH in serum but only with a very high dose of resorcinol administration. The hippocampal anatomy/histopathology was assessed but didn't show any changes. The KEs 3, 4, 6 of *table 4* and the AO of learning and memory deficits were not investigated.

In contrast to the resorcinol studies, the publications investigating the role of PTU on the thyroid and brain have considered more of those KEs. They showed a decrease of TH concentration in serum (Axelstad et al., 2008; Farrokhi et al., 2014; Gilbert et al., 2017; Gilbert

et al., 2014; Goodman & Gilbert, 2007; Hassan et al., 2017; HIASA et al., 1987; Mallela et al., 2014; Spring et al., 2016; Weiss & Burns, 1988) and a decrease in tissues-specific TH concentration (Hassan et al., 2017). Moreover, there was an altered hippocampal anatomy (Gilbert et al., 2017; Gilbert et al., 2014; Goodman & Gilbert, 2007; Li et al., 2004; Niemi et al., 1996; Sawin et al., 1998; Spring et al., 2016) as well as a decrease in hippocampal functioning (Gilbert & Paczkowski, 2003; Niemi et al., 1996; Sui & Gilbert, 2003). However, the results of those two KEs are extracted from different studies, which means that the causality of those can't be confirmed here. There was also a decrease in BDNF seen, although there were no further tests done in relation to neuronal network functioning or cognitive deficits. However, in separate studies increased anxiety and cognitive impairment was observed (Khairinisa et al., 2018) as well as a decrease in learning ability (Farrokhi et al., 2014). Moreover, an impaired context conditioning and cue learning was seen (Gilbert, 2011).

With combination of the results for resorcinol and PTU, all KEs of the TPO inhibition, except for Nr.4 'altered hippocampal gene expression' were observed. However, it would be of importance to investigate if those events are subsequent to one another, in other words if there is causality of those events. For that, studies that investigate all the events of an AOP should be planned and so conclude if a MIE leads to each one of those subsequent outcomes. Moreover, since the data for resorcinol is very limited the weight of evidence to support this AOP is not sufficient and should be considered with caution.

Table 4. Key Events of the AOP #42 for TPO inhibition with sequence of the events, event ID and title of the event based on AOP wiki page. Moreover, it is given which Kes have been assessed in studies of the investigated chemicals of this thesis and which one of those have had an effect on rodents

Sequence	Event ID	Title	Assessed in study of	Observed effect in study
1	277	Thyroid hormone synthesis	resorcinol	resorcinol
2	281	Decreased T4 in serum	PTU, resorcinol	PTU, resorcinol
3	280	Decreased T4 in neuronal tissue	PTU	PTU
4	756	Altered hippocampal gene expression	-	-
5	757	Altered hippocampal anatomy	PTU, resorcinol	PTU
6	758	Altered hippocampal physiology	PTU	PTU

4.3 Sodium-Iodine-Symporter (NIS) inhibition.

Chemicals with similar ion radius as iodide can inhibit the sodium-iodide-symporter (NIS) and hinder its action, which is the iodide uptake from the circulation into the thyroid gland. Based on the AOP #54, 'Inhibition of NIS leads to learning and memory impairment' that has been published on the AOP wiki page, the Kes following that MIE are the ones seen in *table (5)*, which can lead to the adverse outcome of impaired learning and memory. From the 6 studied chemicals in this thesis perchlorate is the one that has shown to be a NIS inhibitor in *in-vitro* studies (Wang et al., 2018). Comparing the results of the 11 relevant studies to the Kes of the known AOP there was a match for two events, namely decreased T4 in serum and a decrease in neuronal network function, more specifically a decrease in baseline synaptic transmission. More Kes that are not mentioned in this AOP but are seen in those studies are an increase of TSH as well as some histopathological changes for example follicular cell hyperplasia and hypertrophy. Moreover, an increased expression of NIS, some transcriptional factors and hypothalamic TSH was observed.

From the 11 studies 7 of them showed some histopathological changes with at least a change in weight of the thyroid gland. Other studies did not asses this endpoint. From that it could be concluded that the histopathological changes could be an additional KE in this AOP. Moreover, the increase of TSH is also a common effect seen in those studies and could be considered an important event for this AOP as it was observed in almost all studies that it was assessed.

The Kes of *table 5* with numbers 1, 2, 4, 5, 6, 7 were not assessed in those perchlorate studies.

The adverse outcome of behavioural changes was assessed but did not show any change (Gilbert & Sui, 2008).

In conclusion, the Kes seen in the *in vivo* perchlorate studies are not sufficient to prove this AOP for this specific substance. It would be of significance to perform studies including all of the mentioned Kes as endpoints in order to have a clearer picture of the presence as well as causality of those events.

Here it should be mentioned that in the studies of resorcinol there is a decreased thyroidal uptake of iodide seen (Cooksey, Doniach, Arnott), which is a characteristic KE of the AOP#54. However, this is not enough evidence to conclude if resorcinol acts as a NIS inhibitor. Moreover, in an *in vitro* study for screening of chemicals for human NIS inhibition resorcinol did not show any effect (Wang et al., 2019).

Table 5. Key Events of the AOP #54 for NIS inhibition with sequence of the event, event ID and title of the event based on AOP wiki page. Moreover, it is given which Kes have been assessed in studies of the investigated chemicals of this thesis and which one of those have had an effect on rodents.

Sequence	Event ID	Title	Assessed in study of	Observed effect in study
1	425	Decrease of thyroidal iodide	-	-
2	277	Thyroid hormone synthesis, decreased	-	-
3	281	T4 in serum, decreased	Perchlorate	Perchlorate
4	280	T4 in neuronal tissue, decreased	-	-
5	381	Reduced levels of BDNF	-	-
6	851	Decrease of GABAergic	-	-
7	385	Decrease of synaptogenesis	-	-
8	386	Decrease of neuronal network function	Perchlorate	Perchlorate

4.4 Deiodinases inhibition

The KEs that have been proposed for the MIE of DIO inhibition are tissue specific altered TH conversions, which lead to an alteration of TH concentration in the tissues and then to an inhibition or activation of gene expression. That can lead to altered hippocampal anatomy and subsequent decrease in hippocampal functioning or a decrease in BDNF and subsequent decrease in synaptogenesis and neuronal network functioning. These events can lead to cognitive, learning and memory deficits (Noyes et al., 2019). There are a number of substances that have shown to inhibit the deiodinases, at least in *in vitro* studies. Here TBBPA, PTU and iopanoic acid have shown to inhibit the deiodinases (Olker et al., 2019).

The KEs observed in the studies for the three different substances and described in the previous chapter have been summarized in *figure 19.* From these results only the KE for 'altered hippocampal anatomy' is shared between TBBPA and PTU. However, as already mentioned there is need for more investigation of the different endpoints to conclude if those KEs are shared between those substances.

It is worth mentioning that in the studies where iopanoic acid was administered there was a T3 decrease and a T4 increase observed, which is different in the studies with the rest of the chemicals, specifically with PTU and TBBPA where both T3 and T4 were always decreased. This effect of iopanoic acid is due to the inhibition of deiodinases and therefore the inhibition of the conversion from T4 to T3 in the different tissues.

The fact that this is not the case with administration of PTU or TBBPA could conclude that the mechanism of deiodinase inhibition is the primary one for iopanoic acid but a secondary mechanism for PTU and TBBPA.

Table 6. Key Events of the AOP Deiodinase inhibition and subsequent adverse outcome of cognitive, learning and memory deficits with sequence of and title of the event based on Noyes et al., 2009. Moreover, it is given which KEs have been assessed in studies of the investigated chemicals of this thesis and which one of those have had an effect on rodents.

Sequence	Title	Assessed in study of	Observed effect in study
1	Tissues specific TH conversion altered	Iopanoic acid	Iopanoic acid
2	Tissues specific TH concentration altered	PTU	PTU
3	Gene transcription altered	-	-
4	Hippocampal anatomy altered	PTU, TBBPA	PTU, TBBPA
	BDNF decreased	PTU	PTU
5	Hippocampal functioning decreased	PTU	PTU
	Synaptogenesis decreased	PTU	PTU

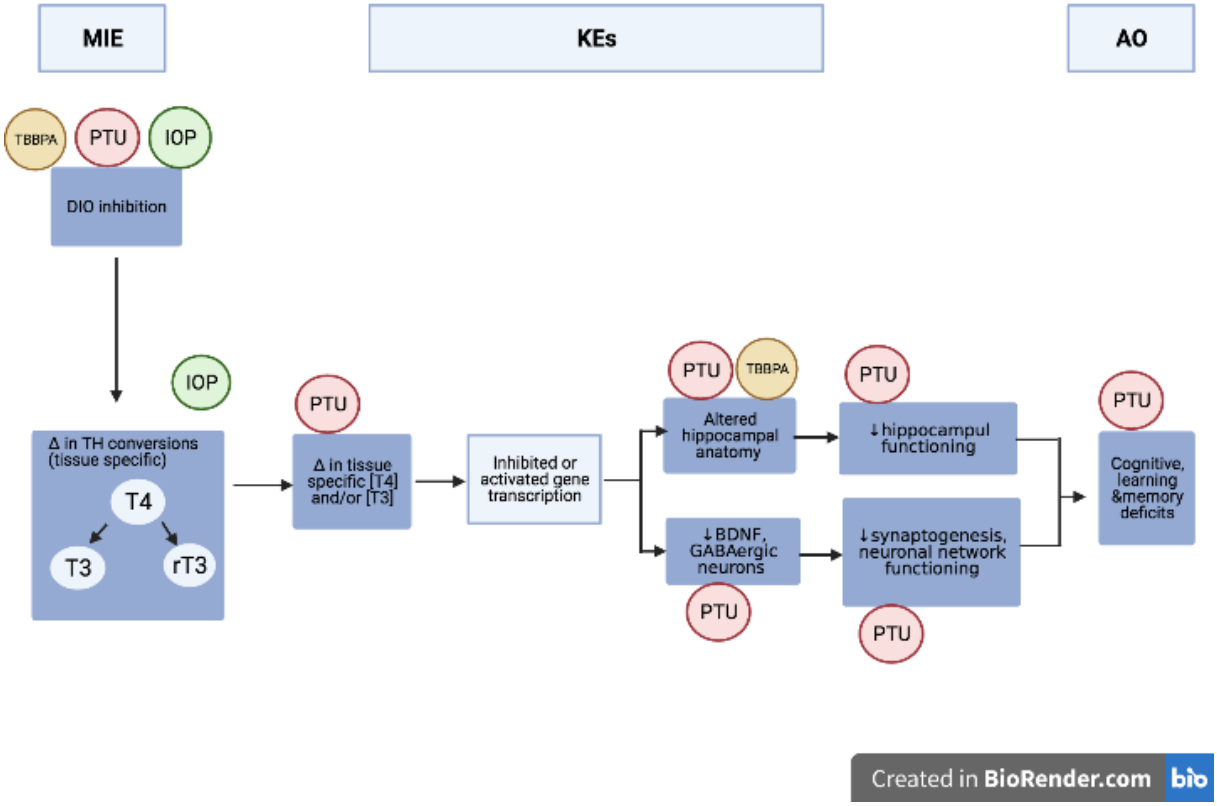

Figure 19. AOP of DIO inhibition with observed KEs in the studies of TBBPA, PTU and iopanoic acid. The observed KE for the substances are given with the yellow, red and green bubbles respectively. The dark blue boxes are events assessed in the available studies of those three substances. The KE in the light blue box is part of the AOP but has not been assessed in those studies.

4.5 Xenobiotic Receptor Activation

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 sulfotransferases (SULTs), respectively, can increase TH catabolism and reduce serum TH by accelerating clearance (Noyes et al., 2019). Here the substance carbamazepine has shown to activate those receptors (Wang et al., 2012). Moreover, there are some indications for liver enzyme induction of the compound TBBPA based on Choi et al. (2011) and Cope et al., 2015. However, since this substance has several different possible MIEs it is not certain, for which mechanism these events are accountable and so cannot be used to strengthen the weight of evidence for this AOP of xenobiotic receptor activation.

The KEs observed in the studies of carbamazepine, which have been analyzed in the results are summarized in *figure 20.*

As already explained in the results the effects of carbamazepine on the anatomy of the brain are contradictory and can't give a coherent understanding of this KE.

The KEs 2, 4, 5, 7 of *table 7* and the adverse outcomes of neurological dysfunction or hearing loss were not assessed in those studies and therefore it cannot be clearly said that based on

those *in vivo* studies these substances follow all the KEs of this AOP or if there is causality between all the KEs. For that and in order to prove the adverse outcome of neurological dysfunction more studies of liver inducing substances should be considered.

There are some events observed in those studies that are not part of the published AOP for xenobiotic receptor activation. Those are the increase in TSH and the thyroid histopathological changes. When carbamazepine was administered six studies assessed the change of TSH, which had all shown a T4 decrease (Ahmed & El-Gareib, 2017; Baumgartner et al., 1994; Baumgartner et al., 1997; Joffe et al., 1988; Oliva et al.) Only two of them showed a TSH increase (Ahmed & El-Gareib, 2017; Oliva et al., 2021). That accounts for 33% of the cases. Therefore, this endpoint is of statistical significance and could be added to the AOP. In general, TSH increase is strongly correlated to the thyroid weight increases and thyroid histopathological effects. For compounds not leading to thyroid effects there is usually no TSH change.

Only one study assessed the histopathology of the thyroid and showed a change in thyroid weight but no morphological changes (Villa & Alexander, 1987). From these results it can't be concluded if this is an endpoint that should be considered in this AOP.

Table 7. Key Events of the AOP #8 for activation of hepatic nuclear receptor and subsequent adverse neurodevelopmental outcomes in mammals with sequence of the events, event ID and title of the event based on AOP wiki page. Moreover, it is given which KEs have been assessed in studies of the investigated chemicals of this thesis and which one of those have had an effect on rodents.

Sequence	Event ID	Title	Assessed in study of	Observed effect in study
1	295	Induction, Upregulation of glucuronyltransferase activity	carbamazepine	carbamazepine
2	401	Increase, Biliary excretion TH glucuronide	-	-
3	281	T4 in serum decreased	carbamazepine	carbamazepine
4	280	T4 in neuronal tissue decreased	-	-
5	756	Hippocampal gene expression altered	-	-
6	757	Hippocampal anatomy altered	carbamazepine	carbamazepine (?)
7	758	Hippocampal physiology altered	-	-

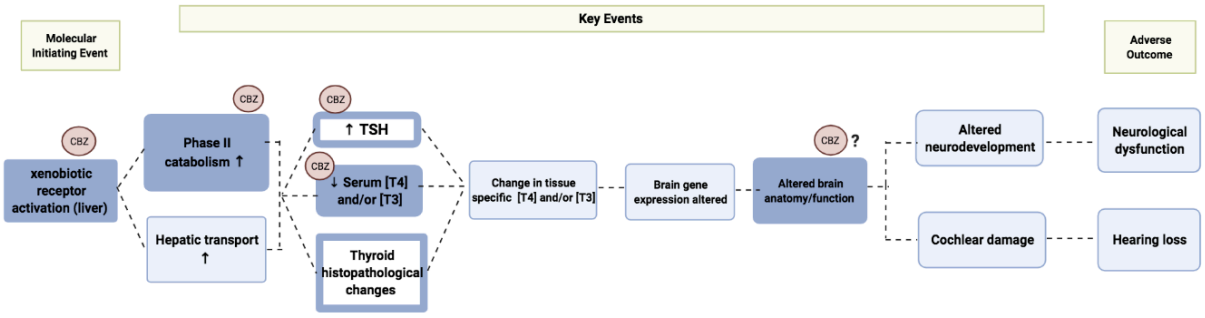

Figure 20. AOP for xenobiotic receptor activation according to AOP wiki, modified with possible additional KEs. The observed KE for the substances of carbamazepine are given with the red bubbles.. The dark blue boxes are part of the published AOP #8 and have been assessed in the available studies. The light blue boxes are part of the AOP #8 but have not been assessed in those studies. White boxes with a dark blue border are not part of AOP #8 but are assessed endpoints in the considered studies.

4.6 Thyroid Hormone Receptor

Chemicals that bind on the thyroid receptors TRα and TRβ can ultimately inhibit or activate the gene transcription with a subsequent altered hippocampal anatomy and function which can lead to cognitive learning and memory deficits. Based on *in vitro* data, TBBPA has shown to have a TR antagonist activity (Paul-Friedman et al., 2019). From the mentioned KEs only the one for altered hippocampal anatomy is observed, whereas the rest haven't been assessed. Also, the adverse outcome of brain deficits has not been assessed.

Table 8. Key Events of the AOP #300 for TR antagonism and subsequent adverse neurodevelopmental outcomes in mammals with sequence of the events, event ID and title of the event based on AOP wiki page. Moreover, it is given which KEs have been assessed in studies of the investigated chemicals in this thesis and which one of those have had an effect on rodents.

Sequence	Event ID	Title	Assessed in study of	Observed in studies
1	756	Hippocampal gene expression altered	-	-
2	757	Hippocampal anatomy altered	TBBPA	TBBPA
3	758	Hippocampal physiology altered	-	-

5 Conclusions

THs are essential for normal human brain development, both prenatally and postnatally, modulating genes critical for a normal neuroanatomical development, with subsequent effects on neurophysiology, and finally neurological function. Therefore, chemicals that can modulate TH concentrations may result in adverse neurodevelopmental effects in offspring (Crofton et al., 2019). In this thesis literature data of rodent studies of six chemicals with known or assumed thyroid modulating effects were evaluated based on *in vivo* rodent studies with main target organs the thyroid, brain and liver. It was evaluated which key events in established or postulated AOPs of TDC were addressed.

After a literature selection procedure, the numbers of eligible publications used for this investigation were: 9 for carbamazepine, 5 for iopanoic acid, 11 for sodium perchlorate, 39 for PTU, 7 for TBBA, and 7 for resorcinol. In those studies, it was seen that all these substances cause a TH change as well as anatomical alterations, for example change in weight of the animals, or histopathological alterations in the target organs. However, some major KEs for the evaluation of the chemical's effect on those organs are missing. For example, the TH measurement in the target tissues as well as brain gene expression (eg. changes in levels of brain-derived neurotrophic factors (BDNF)) and brain functions (eg. synaptogenesis) were not evaluated. Moreover, the adverse outcome of neurological dysfunction, behavioral changes and learning disabilities was only assessed in the studies of PTU and perchlorate and showed an effect only in the studies of PTU. All in all, PTU is the only substance showing clear hippocampal alterations and behavioral changes.

The question if TH changes in serum of maternal rats can provoke neurodevelopmental effects in offspring cannot be answered for the six evaluated chemicals with certainty, because many key events in the established or postulated AOPs are not addressed by published studies so far. Moreover, the following limitations of rat studies evaluating TDCs are discussed:

- Administration of too high doses with the risk of non-thyroid specific effects of the chemicals.
- Confirmation of an AOP by at least two reference compounds to evaluate a thyroid specific, but not a substance specific effect.
- Assessing thyroid hormone levels and deiodinase activity in the target tissue instead of using serum thyroid hormone levels as substitute.
- Evaluation of *in vivo* parameters reflecting neurodevelopmental effects in humans (e.g. special brain histology methods or brain gene expression).
- It is taken as default that effects seen in rats are also relevant for humans. Nonetheless, important species differences (e.g. half-life of T4, serum binding proteins etc.) should be regarded. To confirm human relevance two strategies are discussed:
    - Running studies with other non-rodent species, like fishes, amphibians, (rabbits?) in order to substantiate thyroid modulating effects across vertebrates.
    - Using *in vitro* tests for addressing single key events in the AOPs.
- Introduction of specific time windows for some MIEs and KEs in the AOP in the case of developmental studies.

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7 Supplementary material

Excel table with all the relevant information of the studied publications. In-life parameters, blood/tissue and histopathology parameters are described (only relevant for the electronic version of this thesis).