AOP ID and Title:

AOP 152: Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity
Short Title: Transthyretin interference

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Status

Author status	OECD status	OECD project	SAAOP status
Open for adoption	Under Development	1.41	Included in OECD Work Plan

Abstract

This AOP describes adverse neurodevelopemental effects that may result from xenobiotic interference with thyroid serum binding protein transthyretin (TTR). Binding of TTR by a xenobiotic (the MIE) during certain developmental windows may disrupt the normal neurodevelopment of mammals through a transient increase in free thyroxine (T4) levels, permitting increased tissue uptake of thyroid hormone (TH), followed by a decrease in both serum and neuronal tissue concentrations. Due to the highly conserved nature of the TTR protein, birds, reptiles, fish and amphibians can also express TTR and be impacted by interference by xeniobiotics. The adverse consequences of TH insufficiency depend both on the severity and developmental timing, indicating that exposure to thyroid toxicants may produce different effects at different developmental windows of exposure. This AOP discusses the potential for developmental TTR interference to adversely impact hippocampal anatomy, function, gene expression and, ultimately, cognitive function.

Background

Transthyretin is one of three ancient, highly conserved serum binding proteins that collectively act to transport thyroid hormone (TH) and thus help maintain normal homeostasis via modulation of the hypothalamic/pituitary/thyroid axis. In addition to TTR, albumin (ALB) and thyroxine-binding globulin (TBG) also serve to transport TH in serum and the relative contribution of each binding protein differs across species. In man, TBG has the greatest affinity for thyroxine (T4), followed by TTR and ALB shows the lowest affinity for T4 while prevalence in serum is the opposite, while in rat, TTR is the major serum transport protein (as rats lack TBG). Interference with TH serum binding proteins is one of several mechanisms through which xenobiotics and environmental contaminants can disrupt normal thyroid endocrine function ("thyroid disruptors") and development of this AOP is expected to contribute towards a fuller understanding of the mechanism of TTR interference and how it may be measured *in vitro* as part of a larger screening battery for thyroid toxicants.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	957	Binding, Transthyretin in serum	Binding, Transthyretin in serum
2	KE	958	Displacement, Serum thyroxine (T4) from transthyretin	Displacement, Serum thyroxine (T4) from transthyretin
3	KE	959	Increased, Free serum thyroxine (T4)	Increased, Free serum thyroxine (T4)
4	KE	960	Increased, Uptake of thyroxine into tissue	Increased, Uptake of thyroxine into tissue

Sequence	Type	Event ID	Title	Short name
5	KE	961	Increased, Clearance of thyroxine from tissues	Increased, Clearance of thyroxine from tissues
6	KE	281	Thyroxine (T4) in serum, Decreased	T4 in serum, Decreased
7	KE	280	Thyroxine (T4) in neuronal tissue, Decreased	T4 in neuronal tissue, Decreased
8	KE	756	Hippocampal gene expression, Altered	Hippocampal gene expression, Altered
9	KE	757	Hippocampal anatomy, Altered	Hippocampal anatomy, Altered
10	KE	758	Hippocampal Physiology, Altered	Hippocampal Physiology, Altered
11	AO	402	Cognitive Function, Decreased	Cognitive Function, Decreased

Key Event Relationships

There are no Relationships associated with this AOP

Stressors

Name	Evidence
Halogenated phenols	Low
Polychlorinated biphenyl	High
Polychlorinated dibenzodioxins	Not Specified
Polybrominated diphenyl ethers	High
Isoflavones	High
Perflourinated chemicals	Moderate
Phthalates	Low
Tetrabromobisphenol A	Not Specified
Clonixin	Not Specified
Meclofenamic acid	Not Specified
2,6-dinitro-p-cresol	Not Specified
Triclopyr	Not Specified
2,2',4,4'-Tetrahydroxybenzophenone	Not Specified

Halogenated phenols

Pentachlorophenol is measured in mammals as parent chemical, hexachlorobenzene, and can bind to TTR, like other halogenated phenols (Marchesini et al 2008; van den Berg 1990). Halogenated phenols, such as PCP, have been implicated as thyroid toxicants (Miller et al 2009); however, it appears only 2,4,6-tribromophenol and pentabromophenol can competitively bind TTR (Weiss et al 2015).

Marchesini, G. R., Meimaridou, A., Haasnoot, W., Meulenberg, E., Albertus, F., Mizuguchi, M., … Murk, A. J. (2008). Biosensor discovery of thyroxine transport disrupting chemicals. Toxicology and Applied Pharmacology, 232(1), 150–160. http://doi.org/10.1016/j.taap.2008.06.014

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van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin. Chem Biol Interact 76(1):63–75.

Weiss, J. M., Andersson, P. L., Zhang, J., Simon, E., Leonards, P. E. G., Hamers, T., & Lamoree, M. H. (2015). Tracing thyroid hormone-disrupting compounds: database compilation and structure-activity evaluation for an effect-directed analysis of sediment. Analytical and Bioanalytical Chemistry, 5625–5634. http://doi.org/10.1007/s00216-015-8736-9

Polychlorinated biphenyl

A number of PCBs and metabolites have been found to bind to TTR (Lans et al 1993; Grimm et al 2013; Marchesini et al 2008) and have been frequently found to be associated with interference in thyroid signaling (Boas et al 2012; Gore et al 2015; Miller et al 2009; Murk et al 2013; Preau et al 2015). Overall, the hydroxylated metabolites of PCBs competitively bind T4 more than the parent compounds (Weiss et al 2015). Depending on the congener and/or type of metabolite (i.e. hydroxylate, sulfate, etc.), there are multiple mechanisms by which this class of environmental contaminants can affect thyroid hormone levels in many tissues, including through interfering with TTR-T4 binding and transport of T4 into the brain (Morse et al 1996; Martin and Klaassen 2010; Martin et al 2012). Multiple epidemiological studies in humans have reported associations between exposure to PCBs and/or metabolites and serum thyroid hormone concentrations (Dallaire et al 2009a, 2009b; Eguchi et al 2015), with hydroxylated metabolites demonstrating the greatest potential for TTR interference (Cheek et al 1999; Dirinck et al 2016).

Boas, M., Feldt-Rasmussen, U., & Main, K. M. (2012). Thyroid effects of endocrine disrupting chemicals. Molecular and Cellular Endocrinology, 355(2), 240–248. http://doi.org/10.1016/j.mce.2011.09.005

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Dallaire, R., Muckle, G., Dewailly, É., Jacobson, S. W., Jacobson, J. L., Sandanger, T. M., … Ayotte, P. (2009a). Thyroid hormone levels of pregnant inuit women and their infants exposed to environmental contaminants. Environmental Health Perspectives, 117(6), 1014–1020. http://doi.org/10.1289/ehp.0800219

Dallaire, R., Dewailly, É., Pereg, D., Dery, S., & Ayotte, P. (2009b). Thyroid function and plasma concentrations of polyhalogenated compounds in inuit adults. Environmental Health Perspectives, 117(9), 1380–1386. http://doi.org/10.1289/ehp.0900633

Dirinck E, Dirtu AC, Malarvannan G, Covaci A, Jorens PG, Van Gaal LF. A Preliminary Link between Hydroxylated Metabolites of Polychlorinated Biphenyls and Free Thyroxin in Humans. Int J Environ Res Public Health. 2016 Apr 13;13(4):421. doi: 10.3390/ijerph13040421.

Eguchi, A., Nomiyama, K., Minh Tue, N., Trang, P. T. K., Hung Viet, P., Takahashi, S., & Tanabe, S. (2015). Residue profiles of organohalogen compounds in human serum from e-waste recycling sites in North Vietnam: Association with thyroid hormone levels. Environmental Research, 137, 440–449. http://doi.org/10.1016/j.envres.2015.01.007

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Lans MC, Klasson-Wehler E, Willemsen M, Meussen E, Safe S Brouwer A. 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. Chem Biol Interact 88(1):7–21.

Marchesini, G. R., Meimaridou, A., Haasnoot, W., Meulenberg, E., Albertus, F., Mizuguchi, M., … Murk, A. J. (2008). Biosensor discovery of thyroxine transport disrupting chemicals. Toxicology and Applied Pharmacology, 232(1), 150–160. http://doi.org/10.1016/j.taap.2008.06.014

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Martin, L. A., Wilson, D. T., Reuhl, K. R., Gallo, M. A., & Klaassen, C. D. (2012). Polychlorinated biphenyl congeners that increase the glucuronidation and biliary excretion of thyroxine are distinct from the congeners that enhance the serum disappearance of thyroxine. Drug Metabolism and Disposition: The Biological Fate of Chemicals, 40(3), 588–95. http://doi.org/10.1124/dmd.111.042796

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Polychlorinated dibenzodioxins

Both dioxins and furans have been reported to bind to TTR (Lans et al 1993) and, similar to PCBs, have been reported as thyroid toxicants (Boas et al 2012; Miller et al 2009)

Boas, M., Feldt-Rasmussen, U., & Main, K. M. (2012). Thyroid effects of endocrine disrupting chemicals. Molecular and Cellular Endocrinology, 355(2), 240–248. http://doi.org/10.1016/j.mce.2011.09.005

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Polybrominated diphenyl ethers

It has been shown that many PBDE congeners and/or metabolites (including hydroxylated metabolites of PBDE congeners) can bind to TTR, in some cases more strongly than T4 (Hallgren and Darnerud 2002; Marchesini et al 2008; Ren and Guo 2012; Weiss et al 2015), and this class of congeners has been implicated as a thyroid toxicant (Boas et al 2012; Gore et al 2015; Miller et al 2009; Murk et al 2013). In rats, certain PBDEs have been found to impact learning and memory ability via damage to hippocampal neurons, perhaps through a TTR-mediated transport process as described in this AOP (Driscoll et al 2009; Kato et al 2009; Sun et al 2017). In humans, PBDEs and/or metabolites affect TH during vulnerable windows (i.e. pregnancy) and have been associated with pediatric neurobehavioral development and the developing nervous system, with particular emphasis on the hydroxylated metabolites that have been found to bioaccumulate in serum in children (Athanasiadou et al 2008; Chevrier et al 2010; Dingemans et al 2011; Eskenazi et al 2013; Preau et al 2015).

Athanasiadou, M., Cuadra, S. N., Marsh, G., Bergman, A., & Jakobsson, K. (2008). Polybrominated diphenyl ethers (PBDEs) and bioaccumulative hydroxylated PBDE metabolites in young humans from Managua, Nicaragua. Environmental Health Perspectives, 116(3), 400–408. http://doi.org/10.1289/ehp.10713

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Isoflavones

Soy isoflavones, such as genistein, have been found to bind to TTR (Radovic et al 2006) and have been noted as thyroid toxicants (Miller et al 2009; Murk et al 2013). Synthetic flavonoids, such as EMD 21388 have also been found to interfere with TH transport via TTR binding and have been well-studied in rats (Leuprasitsakul et al 1990; Mendel et al 1992; Pedraza et al 1996; Schröder-van der Elst et al 1997; Schröder-van der Elst et al 1998). This early work in rats helped refine knowledge of the TTR interference pathway and its important to brain and fetal thyroid function.

Lueprasitsakul, W., Alex, S., Fang, S. L., Pino, S., Irmscher, K., Köhrle, J., & Braverman, L. E. (1990). Flavonoid administration immediately displaces thyroxine (T4) from serum transthyretin, increases serum free T4, and decreases serum thyrotropin in the rat. Endocrinology.

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Perflourinated chemicals

Some PFCs have been found to bind to TTR more strongly than T4 and been implicated as thyroid toxicants that could act through transport protein displacement (Boas et al 2012; Coperchini et al 2017; Gutshall et al 1989; Ren et al 2016; Weiss et al 2015; Zhang et al 2015). Zhang et al (2016) determined the X-ray structures of TTR-PFOA and TTR-PFOS. Evidence of interference with thyroid function and/or TH concentrations has been reported in both animals (Chang et al 2008; Yu et al 2009) and human (Ballesteros et al 2016; Dallaire et al 2009; Preau et al 2014; Wang et al 2014), including evidence of transplacental transfer (Yang et al 2016).

Ballesteros, V., Costa, O., Iñiguez, C., Fletcher, T., Ballester, F., & Lopez-Espinosa, M.-J. (2016). Exposure to perfluoroalkyl substances and thyroid function in pregnant women and children: A systematic review of epidemiologic studies. Environment International. http://doi.org/10.1016/j.envint.2016.10.015

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Phthalates

Some phthalates have been found to competitively bind to TTR (Ishihara et al 2003; Weiss et al 2015) and have been implicated as thyroid toxicants (Boas et al 2012; Gore et al 2015; Miller et al 2009). While both animal and human data is scarce, there is some evidence for dose-dependent biochemical and morphological changes in animals (Howarth et al 2001; Liu et al 2015; O'Connor et al 2002) as well as in humans (Huang et al 2007; Meeker et al 2007).

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Tetrabromobisphenol A

Tetrabromobisphenol A (TBBPA) has been found to competitively bind to TTR (Meerts et al 2000; Weiss et al 2015) and other bisphenols have been implicated as thyroid toxicants (Boas et al 2012). Iakovleva et al (2016) recently reported the crystal structure of TTR-TBBPA

Boas, M., Feldt-Rasmussen, U., & Main, K. M. (2012). Thyroid effects of endocrine disrupting chemicals. Molecular and Cellular Endocrinology, 355(2), 240–248. http://doi.org/10.1016/j.mce.2011.09.005

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Clonixin

Similar structure and binding affinity as meclofenamic acid.

Zhang J, Begum A, Brännström K, Grundström C, Iakovleva I, Olofsson A, Sauer-Eriksson AE, Andersson PL. Structure-Based Virtual Screening Protocol for in Silico Identification of Potential Thyroid Disrupting Chemicals Targeting Transthyretin. Environ Sci Technol. 2016 Nov 1;50(21):11984-11993.

Meclofenamic acid

Zhang J, Begum A, Brännström K, Grundström C, Iakovleva I, Olofsson A, Sauer-Eriksson AE, Andersson PL. Structure-Based Virtual Screening Protocol for in Silico Identification of Potential Thyroid Disrupting Chemicals Targeting Transthyretin. Environ Sci Technol. 2016 Nov 1;50(21):11984-11993.

2,6-dinitro-p-cresol

Zhang J, Begum A, Brännström K, Grundström C, Iakovleva I, Olofsson A, Sauer-Eriksson AE, Andersson PL. Structure-Based Virtual Screening Protocol for in Silico Identification of Potential Thyroid Disrupting Chemicals Targeting Transthyretin. Environ Sci Technol. 2016 Nov 1;50(21):11984-11993.

Triclopyr

Major metabolite 3,5,6-trichloro-2-pyridinol has been reported to disrupt TH levels via TTR binding.

Zhang J, Begum A, Brännström K, Grundström C, Iakovleva I, Olofsson A, Sauer-Eriksson AE, Andersson PL. Structure-Based Virtual Screening Protocol for in Silico Identification of Potential Thyroid Disrupting Chemicals Targeting Transthyretin. Environ Sci Technol. 2016 Nov 1;50(21):11984-11993.

Zhang, J., Kamstra, J. H., Ghorbanzadeh, M., Weiss, J. M., Hamers, T., & Andersson, P. L. (2015). In Silico Approach To Identify Potential Thyroid Hormone Disruptors among Currently Known Dust Contaminants and Their Metabolites. Environmental Science and Technology, 49(16), 10099–10107. http://doi.org/10.1021/acs.est.5b01742

2,2',4,4'-Tetrahydroxybenzophenone

Zhang J, Begum A, Brännström K, Grundström C, Iakovleva I, Olofsson A, Sauer-Eriksson AE, Andersson PL. Structure-Based Virtual Screening Protocol for in Silico Identification of Potential Thyroid Disrupting Chemicals Targeting Transthyretin. Environ Sci Technol. 2016 Nov 1;50(21):11984-11993.

Overall Assessment of the AOP

Essentiality of the Key Events

Molecular Initiating Event Summary, Key Event Summary
Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

In vivo evidence for MIE

Kohrle et al (1989) added 10 µmol/L 3-methyl-4',6-dihydroxy-3',5-dibromo-flavone (EMD 21388) to pooled rat serum and measured displacement of [125I]-T4 from TTR. EMD21388 was synthesized using "molecular drug design" (and resembles T4) to help confirm previous findings that certain flavonoid deiodinase inhibitors also displaced thyroxine (T4) for TTR (or T3-binding prealbumin). Displacement of [125I] from TTR in rat serum was analyzed by gel electrophoresis (PAGE) and individual serum samples were assayed for T3 and T4 content by RIA and % free TH by equilibrium dialysis (lower limit of detectability 0.3 ug/dL for T4). There was a significant increase in % free T4 (0.031 to 0.124), which was dose-dependent and resulted in complete inhibition of [125I]-T4/TTR at 8-10 umol (radiolabeled TH were displaced primarily to albumin).

insert Fig 2 from Kohrle et al 1989

One to 4 hours following ip delivery of 2 µmol/100 g BW to euthyroid Sprague-Dawley rats (a dose that is 1000x higher than daily T4 production in rat), inhibition of [125I]-T4/TTR binding was observed. T4 decreased from 5.6 to 2.3 ug/dl after 1 hour and remained low while % free T4 increased from 0.035 to 0.091 and remained high; however, free T4 did not change. TSH decreased to very low values after 2 hours and increased slightly, despite no change in the free TH concentration (hypothyroid rats did not show changes in serum TSH following EMD 21388 administration). Lueprasitsakul et al (1990) performed a series of experiments with Sprague-Dawley rats using smaller doses of EMD 21388 (up to 2 µmol /100 g BW) and the same measurement methods (RIA, equilibrium dialysis). Administration of 2 µmol of EMD 21388 inhibited [125I]-T4/TTR binding within a few minutes, displacing [125I] to albumin to a greater degree of magnitude, due to slight differences in preparing the EMD 21388 solutions. Dose-dependent decreases in displacement were found with decreasing dose.

insert Figure 1 & Figure 2

Following a single dose of 2 µmol, a significant decrease was seen in total serum T4 after 10 minutes that persisted, % free T4 also increased immediately (peaked after 10 minutes) and stayed elevated and a significant increase in free T4 was observed within three minutes that stayed elevated for 60 minutes. Following a single dose of 0.3 µmol, decreased [125I]-T4/TTR binding was observed reaching a nadir after 10 minutes and slowly recovering over the 180-minute experiment. The % free T4 and serum free T4 both increased and returned to normal after 180 minutes as well while total serum T4 hit a nadir after 10 minutes and mostly recovered. Serum TSH decreased after 20 minutes, significantly at the nadir hit after 60 minutes.

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Appendix 1

List of MIEs in this AOP

Event: 957: Binding, Transthyretin in serum

Short Name: Binding, Transthyretin in serum

Key Event Component

Process	Object	Action
binding	transthyretin	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	MolecularInitiatingEvent
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	MolecularInitiatingEvent

Stressors

Name
Halogenated phenols
Polychlorinated biphenyl

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
serum

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Rickenbacher et al (1986) provided initial direct evidence of competition for the T4 binding site using molecular modeling and binding assays using radiolabeled TH. Brouwer and van den Berg (1986) reported preferential binding of a metabolite of radiolabeled tetrachlorobiphenyl to TTR in rats (15 mg/kg, ip), using gel electrophoresis followed by HPLC analysis. Van den Berg (1990) used a competitive binding assay to assess the ability of hydroxylated chlorinated aromatic compounds to bind to radiolabeled T4. Van den Berg et al (1991) extended this work to 65 compounds from 12 different chemical groups in rats treated via a single ip dose and competitive binding assay. Chlorophenols were found to have higher affinity relative to other chlorinated aromatics, particular at higher levels of chlorination, and the combination and position of hydroxyl & chlorine atoms. {insert Figure 2/Van den Berg 1990}

Lans et al (1993) described the ability of hydroxylated metabolites of PCBs, PCDDs and PCDFs to act as competetive ligands at the TTR-T4 binding site using an in vitro binding assay. Many of the hydroxylated PCBs examined were more potent ligands than T4, as opposed to those PCDFs and PCDDs that lacked chlorine atoms substituted adjacent to hydroxyl groups. When the hydroxyl group was in the meta or para positions, a 35- to 136-fold greater potency was found relative to ortho substitutions. These results were confirmed through later competitive binding work published by Cheek et al (1999) and Chauhan et al (2000).

Weiss et al (2009) first confirmed competitive binding of TTR with perfluorinated compounds (PFCs)

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Bubalus bubalis	Bubalus bubalis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Gallus gallus	Gallus gallus	High	NCBI
Sus scrofa	Sus scrofa	High	NCBI
Pan troglodytes	Pan troglodytes	High	NCBI
Monodelphis domestica	Monodelphis domestica	High	NCBI
Xenopus tropicalis	Xenopus (Silurana) tropicalis	High	NCBI
Bos taurus	Bos taurus	High	NCBI
Macaca mulatta	Macaca mulatta	High	NCBI
Meleagris gallopavo	Meleagris gallopavo	High	NCBI
Canis lupus familiaris	Canis lupus familiaris	High	NCBI
Ovis aries	Ovis aries	High	NCBI
Erinaceus europaeus	Erinaceus europaeus	High	NCBI
Xenopus laevis	Xenopus laevis		NCBI

The transthyretin protein has been found to be highly conserved among vertebrates, as supported by X-ray crystallography studies in man, rat, chicken and fish as well as comparison of amino acid sequence (Richardson 2007). Over time, evolution has driven the nature of the N-terminal region of TTR from a more hydrophobic and longer region (as found in fish and amphibians) to a shorter and more hydrophilic region (as found in the placental mammals). The consequence of this biochemical change in the TTR protein was to change the primary function of TTR from binding and carrying T3 in serum to carrying T4 in serum (as it does almost exclusively in rats, as rats lack TBG during adult life)(Alshehri et al. 2015). Thus, in the placental mammals, TTR operates to carry T4 to specific tissues, where it is displaced, transported across the cell membrane by a different ligand-mediated process, and then converted to T3 via deiodinase enzymes within the cell (where it activates the nuclear receptor to cause downstream effects).

TTR is encoded by a single gene on human chromosome 18 (18q11.2-12.1) (LeBeau and Geurts van Kessel 1991).

TTR is synthesized in the adult liver only by eutherians (birds and placental mammals) and herbivorous marsupials; however, it is also synthesized in the choroid plexus of reptiles, birds and mammals. TTR is one of three thyroid hormone serum transport proteins, along with albumin (ALB) and thyroid-binding globulin (TBG). Over evolutionary time, the N-terminal section of TTR has become shortened and more hydrophilic such that T3 is more tightly bound in birds and reptiles but T4 is more tightly bound to TTR in mammals (Schrieber 2002). They all differ in binding affinity and dissociation rate for both T4 and T3 and together form a buffered system that seeks to keep circulating T4 within a certain range: between the normal concentration of free T4 in serum (30 pM) and solubility limit in serum (2 uM) (Schreiber 2002). Given these differences, more T4 available for cellular uptake likely comes from ALB-bound T4 in capillaries with fast moving blood but TTR-bound T4 is likely more important in slow moving fluid (like CSF)(Schrieber 2002).

Mammalian TTR has a greater affinity for T4 (and lower affinity for T3) in mammals relative to birds and reptiles, neither of which express a high-affinity TBG protein in serum (Schweizer and Kohrle 2013); while Larsson reported the presence of TTR (i.e. thyroxine-binding prealbumin) in all vertebrate species investigated and failed to detect the presence of TBG in cat, rabbit, rat, chicken, frog and salmon. Binding capacity of serum TTR in female rats is lower relative to males (Emerson et al 1990 in Zoeller et al 2007).

Key Event Description

The key event that initiates this AOP (i.e. the MIE) is binding of a xenobiotic to the thyroxine (T4)-binding site of transthyretin (TTR), displacing T4 from the binding site(s) of TTR and adding this to the pool of free T4 in serum, which is normally roughly 0.02% (20 pM) of total T4 (100 nM) [in CSF, free T4 is roughly 1.4% (70 pM) of total T4 (2-3 nM)](Schweizer and Kohrle 2013).

TTR has been found to bind with a number of ligands, including pharmacologic agents as well as flavonoids and halogenated aromatic compounds, with differing strengths with some xenobiotics found to be possessing a binding affinity equal to or stronger than T4 (i.e. "competitive binder"). Studies with other xenobiotics with similar structural characteristics have found that a higher degree of halogen substitution, as well as placement on the ring relative to the hydroxyl group, increases binding affinity (Chauhan et al. 2000, Lans et al. 1993, Lans et al. 1994, Ren and Guo 2012, van den Berg 1990, van den Berg et al. 1991, Weiss et al 2015). Weiss et al (2015) compiled a database of almost 150 compounds found to bind to TTR and 52 of these were found to have higher affinity than T4 and thus, could be considered competitive binders in serum.

The function of the serum protein TTR is to deliver T4 to target cells in the liver, tight junctions, etc. where it is facilitated across the membrane via specific receptors and converted to the active form T3, where it can activate nuclear receptors. TTR facilitates passage across key tight junctions, such as the blood-brain barrier, the CSF barrier and transplacentally, and the interruption of thyroid serum protein-assisted transport during certain developmental windows can lead to profound developmental neurotoxicity (i.e. cretinism). It should be noted, though, that in mammals with all three functional serum transport proteins (TTR, albumin and TBG), substantial reductions in total T4 can be observed with little to no adverse effect due to overall redundancy of this system. In

this scenario, roughly 75% of serum T4 is bound to TBG, 15% to TTR and up to 5% for to albumin (OECD DRP 2012). That being said, TTR is the sole transport protein in the CSF and T4 is not biosynthesized by the fetus until the second trimester - thus, the mother is the sole source of T4 for the fetus during early gestation and this appears to be the developmental window of greatest concern to human physiology (for example, see Loubiere et al 2010).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Transthyretin is a 55-kDa tetramer protein composed of two identical monomer subunits, each of which is formed by two four-stranded beta sheets, which are assembled around the central channel of the protein (Blake et al. 1978). This central channel contains two identical binding sites; however, there is a 100-fold difference in binding constants between the first and second T4 molecule bound and this "negative cooperativity" results in a 1:1 relationship between T4 and TTR in terms of protein transport in serum (Ferguson et al. 1975). The phenolic hydroxyl and iodine substitutions on the phenolic ring of T4 are important structural characteristics that differentiate ability to bind to TTR, as opposed to other serum binding proteins like TBG (Andrea et al. 1980). T4 binds to TTR in one of two ways: "forward" and "reverse" mode. In forward mode, the phenolic ring of T4 is buried deeply in the TTR binding site while this ring interacts with residues at the entrance of the binding channel in reverse mode (Lans et al 1993). Detailed structural analysis of the protein is available from X-ray crystallography (Rerat and Schwick 1967; Blake et al 1977).

There are three main *in vitro* approaches to measuring binding affinity between TTR and its ligands: radioligand-binding assays (RLBAs), surface plasmon resonance (SPR) and fluorescence displacement. Biochemical (*in chemico*) approaches using GC/MS or LC/MS also exist that use recombinant (rTTR) and display limits of detection in the low nM range, including a multi-mode, ultra-high-performance LC method and anon-radioactive label, [$^{13}C_6$]-T4 (Aqai et al 2012).

Van den Berg et al (1991) used an in vitro competitive radioligand binding assay with gel-filtration procedure modified from Somack et al (1982) as well to screen 65 compounds from 12 chemical classes for evidence of interference with the T4 binding site of TTR using a radioligand binding assay and reported results semi-quantitatively, while previous work using the same method on chlorinated phenols found that affinity rose with degree of chlorination (pentachlorophenol displayed an IC50 of 2.3 x 10^8 M; derived K_a = 1.2 x 10^8 M^{-1}) versus the natural ligand (T4; 4 x 10^8 M)(Van Den Berg, 1990).

Lans et al (1993, 1994) used purified TTR and T4 [^{125}I] as a displaceable radioligand and gel-filtration procedure modified from Somack et al (1982) to study hydroxylated PCBs PCDDs and PCDFs and found many competitive binders in all classes; again, dependent on degree of chlorination, presence and placement of hydroxyl atom relative to chlorine placement (IC50 in 6.5-25 nM range; K_a = 0.78-3.95 x 10^8 M^{-1}) versus the natural ligand (T4, K_a = 2.05 x 10^7 M^{-1}). Meerts et al (2000) studied PBDEs and related compounds (pentabromophenol, Tetrabromobisphenol A) and found multiple competitive binders (IC50 range 7.7 - 67 nM; Ka = 4.3 - 38 x 10^7 M^{-1}) versus the natural ligand (T4; IC50 = 80.7 nM; Ka = 3.5 x 10^7 M^{-1}).

Cao et al (2010) used a novel fluorescence displacement method using a protein-binding probe that fluoresces when bound, and the intensity of which dips following displacement for a ligand. Analyte titration curves are used to derive IC50 and binding constant values. They used an ANSA probe to screen 14 hydroxylated PBDEs and found competitive binding with TTR (K_a = 1.4 x 10^7 M^{-1} to 6.9 x 10^8 M^{-1}). Molecular docking analysis revealed a ligand-binding channel in TTR for OH-PBDE binding. Ren and Guo (2012) reported use of a non-radioactive, fluorescin-T4 conjugate designed as a fluorescent probe (vs ANSA probe which is less TTR-specific) to study the binding interaction of multiple hydroxylated PBDEs with differing degrees of bromination and differing hydroxy positions and TTR. Competitive binders were found (IC50 range = 110-219 nM) relative to the natural ligand (T4; IC50 = 260 nM) and Kd values for the competitive binders ranged from 101-210 nM versus T4 (Kd = 239 nM). Weiss et al (2009) initially determined the competitive binding of TTR with PFCs via radioligand-binding assay of Hamers et al (2006) and Lans et al (1993). Ren et al (2016) used a fluorescence displacement assay to study 16 PFCs and found T4 to have an IC50 of 31 nM (Kd = 5 nM), as compared to PFOS (the strongest binder) TC50 of 130 nM (Kd = 20 nM).

The radioligand methods have been criticized for use of hazardous and costly radio-labeled material and physical separation of bound and free T4-[^{125}I] is required. Marchesini et al (2006, 2008) reported a surface plasmon resonance biosensor-based method using rTTR, a surface-based measurement which may not fully characterize the binding reaction of a homogenous solution or matrix. Optimized SPR is more sensitive, amenable to high-throughput screening and easier to perform than RLBAs. Furthermore, hydroxy-metabolites do not endure destructive clean-up/extraction methods and differences in physicochemical qualities from parent compounds complicates separation.

Montano et al (2012) introduced a new method (using a modified method of Murk et al 1994 for in vitro bioactivation of PCBs and PBDEs with an ANSA-TTR displacement assay) to selectively extract metabolites (and limit co-extraction of interferants, like free fatty acids) and derive an IC50 dose-response curve for OH-PBDEs, OH-PCBs, etc. using bioactivated parent compound in a 96-well plate system. IC50 for the natural ligand (T4) was 0.26 uM while the IC50s for the hydroxy metabolites ranged from 0.23-0.63 uM.

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List of Key Events in the AOP

Event: 958: Displacement, Serum thyroxine (T4) from transthyretin

Short Name: Displacement, Serum thyroxine (T4) from transthyretin

Key Event Component

Process	Object	Action
	thyroxine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent

Stressors

Name
2,4-Dinitrophenol

Biological Context

Level of Biological Organization
Molecular

Key Event Description

Despite the two binding sites for T4 on the TTR serum binding protein, each molecule of TTR only carries a single T4 molecule due to the negative cooperativity displayed by these binding sites (Ferguson et al 1975). As such, xenobiotics and pharmacologic agents can displace T4 from TTR and early on, this was demonstrated for ethnacrynic acid, salicylates, penicillin and 2,4-dinitrophenol (Munro et al 1989). More recently, work with the flavonoid EMD 21388 (and other compounds structurally similar to thyroxine) showed competitive binding and displacement of T4 from the TTR carrier protein.

Rickenbacher et al (1986) provided initial direct evidence of competition for the T4 binding site using molecular modeling and binding assays using radiolabeled TH. Brouwer and van den Berg (1986) reported preferential binding of a metabolite of radiolabeled tetrachlorobiphenyl to TTR in rats (15 mg/kg, ip), using gel electrophoresis followed by HPLC analysis. Van den Berg (1990) used a competitive binding assay to assess the ability of hydroxylated chlorinated aromatic compounds to bind to radiolabeled T4. Van den Berg et al (1991) extended this work to 65 compounds from 12 different chemical groups in rats treated via a single ip dose and competitive binding assay. Chlorophenols were found to have higher affinity relative to other chlorinated aromatics, particular at higher levels of chlorination, and the combination of hydroxyl chlorine atoms in the ortho position. {insert Figure 2/Van den Berg 1990}

Kohrle et al (1989) showed complete displacement (via gel electrophoresis) of radiolabeled T3 and T4 by EMD 21388 in pooled rat serum followed by increase in the percent free TH as measured by equilibrium dialysis. Complete inhibition occurred at 10 umol and displaced T4 from TTR to serum albumin and thyroxine-binding globulin (TBG), which normally serve a lesser role in thyroid hormone transport in humans. {insert Figure 2}

Kohrle et al (1989) administered EMD 21388 via ip route to rats at 2 umol/100 g BW and observed displacement of radiolabeled T3 and T4 from TTR followed by a decrease of T3 and T4 in serum while the percent free TH remained unchanged. {insert Figures 3-5}

Lueprasitsakul et al (1990) repeated this protocol and found that inhibition of binding occurred within 3 minutes followed by a decrease in serum T4 concentration and an increase in both serum percent free T4 as well serum total T4. {insert Figures 1 and 4}

Mendel et al (1992) demonstrated in rats dosed via IP with EMD 21388 (2 µmol/100 g BW) both displacement of radiolabeled T4 from TTR (as assessed via electrophoresis of serum proteins) and susbsequent increase of free T4 in serum.

To initially evaluate the impact of EMD 21388 on maternal/fetal hormones, Pedraza et al (1996) administered 2.5 mg 21388/day subcutaneously in pregnant female rats which led to displacement of T4 from TTR, reduced total T4 and increased free T4 in maternal circulation.

Compounds that have been found to compete with TTR for binding to T4 (and thus lead to some degree of thyroid disruption) include pharmaceuticals and environmental contaminants, such as halogenated aromatic compounds. This latter category include PCBs, PBBs, PBDEs and perfluoro compounds and specifically, hydroxylated metabolites of all these compounds often display greater binding affinity than the natural ligand; however, this is a function of degree of halogenation as well as orientation of the

halogens and hydroxyl functional group.

Gutshall et al 1989 treated male Wistar rats with a single IP dose of perfluorodecanoic acid (PFDA) and ^{125}I and then measured TH, uptake of ^{125}I, liver enzymes and binding of [^{125}I]-T4 to albumin. The authors did not observe increased conversion of T4 to rT3 but did note that PFDA displaced [^{125}I]-T4 from rat albumin with an affinity similar to T4. While this study involved albumin, it showed that perfluoro compounds may also have potential to interact with thyroid serum transport proteins.

Meerts et al 2000 investigated the affinity of several polybrominated flame retardants (including 17 PBDE congeners) to TTR using human TTR in an in vitro competitive binding assay and [^{125}I]-T4. Pentabromophenol and tetrabromobisphenol A were found to have affinities 7-10 fold that of T4; however, a microsomal enzyme mediated transformation was needed first (i.e. hydroxylation) for PBDEs. It should be noted that no reference OH-PBDEs were available at the time of this experiment. Like the PCB congeners, degree of bromination is a driver of binding potency as is the nature of the halogen substitution (as well as hydroxy substitution). In addition, brominated analogs are more potent in general relative to chlorinated ones. Hydroxylation of parent compound via CYP2B enzymes appears to be a prerequisite of binding to TTR.

Hallgren et al 2001 treated female Sprague Dawley rats and C57BL/6 mice daily with Aroclor 1254, PCB-105, Bromkal 70-5 DE (commercial PBDE mixture) or BDE-47 via gavage for 14 days and measured TH, induction of microsomal phase I enzymes (EROD, MROD, PROD) and UDP UGT activity. Free and total T4 was decreased in both species with no significant change to TSH and minimal impact on UDP UGT activity. Rats were found to be more sensitive than mice to the observed effects. The findings suggested that PBDEs may be metabolized by CYP2B, but also CYP1A to an extent, and that these induced enzymes increased the availability of hydroxylated metabolites *in vivo* and increase binding to T4 transport proteins.

Hallgren and Darnerud 2002 treated female Sprague Dawley rats with BDE-47, Aroclor 1254 and Witaclor 171P, alone or in combinations, daily via gastric intubation for 14 days. Microsomal enzyme (cytochrome P450 isozymes and UDP UGTs), ex vivo binding of [^{125}I]-T4 to plasma proteins and light microscopy morphology of the thyroid was examined. Aroclor 1254 and BDE-47 was observed to decrease T4, decrease [^{125}I]-T4 binding to TTR, induce several phase I enzymes as well as moderate elevation of UDP UGT activity. These data suggested that decreased plasma T4 is mainly due to interference with serum transport binding of parent and metabolites to TTR; however, there was clearly some role for glucuronidation in reducing T4 in this study. (PCB mixtures have been demonstrated to impact a number of different endpoints affecting normal thyroid homeostasis.)

Metabolites of BDE 47 formed by CYP2B6 include hydroxylated BDE 47 in addition to other hydroxylated congeners (Erratico et al 2013, Feo et al 2013) and it should be noted that CYP2B6 is also expressed in brain which has implications for formation of hydroxylates in that tissue (Miksys and Tyndale 2004).

Cao et al 2004 performed molecular docking analysis on the TTR and OH-PBDE interactions and confirmed the effect of degree of bromination.

Darnerud et al 2006 treated female Sprague Dawley rats with BDE 47 or Bromkal 70-5 DE at 2 doses via gavage daily for 2 weeks. Thyroid hormones were measured from plasma via radioimmunoassay and samples pooled for analysis for individual congeners (BDEs 28, 47, 66, 99, 100, 138, 153, 154) and internal plasma doses were calculated that corresponded with decreased free T4. This critical dose was estimated to be ~ 400 ug/g lipid BDE 47 based on significant reduction of free T4.

Hamers et al 2006, 2008 collectively found that OH-PBDEs act as agonists or antagonists at TH receptors, that OH-PBDEs with high affinity for T4 can be detected in human serum and all metabolites were found to be more potent than the natural ligand in vitro using rat liver microsomes (3-OH-BDE-47 was found to have the highest potency).

Lau et al 2007 and Chang et al 2008 reported that PFOS alters serum T4 via interference with binding proteins, leading to a transient increase in free T4 and decrease in TSH.

Weiss et al 2009 were the first to examine the potential of 24 perfluorinated compounds and 6 structurally-related fatty acids to compete with T4 for TTR via [125I]-T4 binding assay and HPLC-MS/MS analysis. From this analysis, 56 chemical descriptors to evaluate the structure-activity relationship (SAR) of binding potency of perfluorinated compounds to TTR. Binding potency was found to strengthen with degree of fluorination, with maximum potency found at a chain length of eight (8) carbons; however, in general, the perfluorinated compounds were found to have T4 binding potency at about 10% that of the natural ligand.

Cao et al 2010 looked at binding interactions for 14 OH-PBDEs with TTR and TBG using fluorescence probe & competitive binding assay, circular dichroism (spectroscopic measurement of a protein's secondary structure) and molecular docking analyses. Binding constant data was generated for the first time and affinity was observed to increase with degree of bromination, until a peak at the 5- and 6-brominated diphenyl ethers was reached. CD analysis showed that the OH-PDBEs bind to TTR and TBG at the same sites as the natural ligand while the molecular docking studies revealed a ligand-binding channel in TTR that was mostly hydrophobic inside but characterized by a positive-charged Lys15 residue at the channel entrance. The novel binding constant allowed meaningful quantitative evaluation of competitive displacement, by assuming human serum levels reported in the literature (Athanasiadou et al 2008; Marchesini et al 2008) and choosing the congener with the highest potency (5-OH-BDE47). This evaluation suggested that competitive displacement would be insignificant, as serum levels of protein-bound 5-OH-BDE47 are at least two orders of magnitude lower than protein-bound T4 (suggesting ~10% T4 displacement by 5-OH-BDE47).

Cao et al 2011 generated binding constant data for the interactions of BPA and TH for TTR, TBG and human albumin via fluorescence probe, noting that concentrations of BPA commonly reported in human plasma are likely not high enough to interfere with T4 transport in serum. A large excess of TTR and albumin in plasma are found relative to T4 and BPA and there is no competition for binding, which is further supported by the fact that affinity of BPA for T4 is much weaker than the natural ligand (by

2-3 orders of magnitude).

Ren and Guo 2012 designed a fluorescin T4 conjugate for use as a fluorescence probe in binding assays to examine interaction between eleven OH-PBDEs and transport proteins TTR and TBG. 3-OH-BDE47 and 3'-OH-BDE154 were found to be competitive with T4.

Ren et al 2013 higher brominated OH-PBDEs act as antagonists (i.e. BDEs 154 and 188) while lesser bromination (i.e. BDEs 47) found to be agonists

Grimm et al 2013 used fluorescence probe displacement and molecular docking simulations to characterize the binding of sulfated PCB metabolites to TTR, and stability and reversibility of these complexes were characterized by HPLC. The hydroxylated PCB metabolites (OH-PCBs) are excellent substrates for sulfation (phase II conjugation) via sulfotransferases (SULTs) and thus could represent another mechanism through which clearance can occur. Of the five lower-chlorinated sulfates for which K_d values were generated and compared against T4, only one would be considered a competitive inhibitor (4'PCB 11 sulfate) and the only case where the sulfate displays higher affinity than its corresponding OH metabolite (4' OH-PCB 11). Molecular docking simulation confirmed the affinity that PCB sulfates have for TTR, confirming previous reports showing higher affinity among those congeners with meta- and para-chlorination. These data demonstrate the toxicological relevance of PCB sulfates to TTR-mediated transport of thyroid hormones in serum for the first time. The generation of sulfates from OH-PCBs could be another mechanism through which PCBs may disrupt thyroid homeostasis.

Weiss et al 2015 compiled a database of 250 compounds and mixtures (including 33 never tested before), of which 144 were TTR binders and 36% (n=52) of these were found to be more potent than the natural ligand T4. The vast majority of these 52 (n=48) were aromatic, halogenated and hydroxylated. A subset of 220 compounds was further analyzed via PCA and a set of chemical descriptors to understand the chemical characteristics of TTR binders and four significant components were found to explain 85% of the variance.

Zhang et al 2015 developed a QSAR model and applied to a database of almost 500 dust contaminants taken from literature data and over 400 in silico derived metabolites, predicting 37 contaminants and 230 metabolites as potential TTR binders. Twenty-three (23) contaminants were than analyzed via radioligand binding assay which identified four novel TTR ligands that were then analyzed via molecular docking studies.

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

In humans, approximately 0.03% of total serum T4 is present in unbound/free condition (Refetoff et al 1970). Of the bound T4, approximately 75% is bound to TBG, approximately 20% to TTR and the remainder to ALB and some high density lipoprotein carriers. ALB is present at roughly 100-fold the molar concentration of TTR and roughly 2,000-fold higher than TBG; however, the affinity of T4 to TBG is 50-fold higher than TTR and 7,000-fold higher than ALB (Refetoff 2015). TTR binds roughly 80% of the T4 circulating in ventricular CSF although it constitutes only 25% of protein found there (Herbert et al 1986). In serum, only about 0.5% of circulating TTR is bound to T4, average serum concentration is 25 mg/dL (which can bind up to 300 ug T4/dL (Refetoff 2015).

TTR can be measured by densitometry after its separation from other serum proteins via electrophoresis, hormone saturation and/or immunoassays (Refetoff 2015).

Total T4 is most often measured using human serum based diagnostic kits, but free T4 (and T3) is only directly measured through equilibrium dialysis and ultrafiltration (Midgley 2001). Large volumes of serum must be used due to the very low concentrations of free T4 normally found (0.1% of total T4), which requires pooling of samples taken from fetus or pup. Some researchers have tried to "micronize" this process through combining RIA to measure total TH and dialysis to estimate the free fraction (Zoeller et al 2007). Extracted materials can also be quantified by HPLC. The reference range for free T4 is 9.8 to 18.8 pM/L (Dirinck et al 2016).

T3 is found in similar plasma concentrations to T4 (i.e. 5-10 pM) with < 0.4% being in the unbound state. Measuring free serum T3 is labor intensive and requires equipment not available in many clinical reference laboratories and thus ultrafiltration is often used (Abdalla and Bianco 2014). Immunoassays and MS/MS are also used.

Measuring displacement of T4 from serum transport proteins is done mainly via one of three *in vitro* methods: radioligand binding assay, plasmon resonance-based biosensor, or fluorescence displacement.

Radioligand binding assays, using [^{125}I]-T4 as a label, were developed to demonstrate affinity for xenobiotics to human or rat TTR and TBG (Brouwer and van den Berg 1986, Lans et al 1994). The most commonly used method was first published by Somack et al 1982 and adapted by Hamers et al 2006, Lans et al 1993 and Ucan-Marin et al 2010. Similar assays have been developed using [^{125}I]-T3 as a label for affinity to chicken and bullfrog TTR (Yamauchi et al 2003). Radioligand methods suffer from having to use heavily regulated isotopes and lower throughput to provide free T4 measurements (due to the extra wash/separation procedure needed). The most well-known protocol uses TTR purified from human serum (which may not be as stable as recombinant) and performed in a pure aqueous solution, which may not be as stable for lipophilic compounds (Chauhan et al 2000 is an example using PCBs).

Purkey et al 2001 published a binding assay using polyclonal TTR antibodies covalently bound to sepharose resin which is then mixed with plasma pre-treated with compound of interest, washed and analyzed via HPLC.

Marchesini et al 2006 reported on the development of two surface plasmon resonance(SPR)-based biosensor assays using recombinant TTR and TBG, validated with known thyroid disruptors and structurally related compounds including halogenated phenols, polychlorinated biphenyls, bisphenols and a hydroxylated PCB metabolite (4-OH-CB 14). TH is covalently bound to a gold-layered chip and a mixture of the compound of interest and transport protein are injected in a flow cell passing over the bound TH. The authors found that these biosensor methods were more sensitive (IC50 of 8.6 ± 0.7 nM for rTTR), easier to perform and more rapid that radioligand binding assays and immunoprecipitation-HPLC.

Marchesini et al 2008 applied their biosensor-based screen to 62 chemicals of public health concern and found that hydroxylated metabolites of PCBs (particularly para-hydroxylated ones) and PBDEs (BDEs 47, 49 and 99) displayed the most potent binding to TBG and TTR, confirming many other previous studies. The authors conclude their optimized assays are suitable for high-throughput screening for potential thyroid disruption.

Cao et al 2010, Cao et al 2011 and Ren and Guo 2012 developed the FLU-TTR, based on a protein-binding fluorescent probe (ANSA, or 8-anilo-1-naphthalenesulfonic acid ammonium salt) that becomes highly fluorescent after binding to T4. When the compound of interest is introduced and displaces the ANSA-thyroxine probe, this fluorescence is reduced. This allows generation of binding constant (K) data as opposed to past efforts that generated IC_{50} values. Cao et al 2011 developed a fluorescent microtiter method for pTTR and TBG tested with bisphenol A.

Montano et al 2012 developed a competitive T4-TTR fluorescence displacement assay in a 96-well format, modified from the original method (Nilsson and Petersen 1975) and using a new selective method to extract hydroxylated metabolites while reducing fatty acid interference (modified from Hovander et al 2000).

Aqai et al 2012 described a rapid and isotope-free ($^{13}C_6$-T4) screening of thyroid transport protein ligands, using a competitive binding assay for rTTR using fast ultrahigh performance LC-electrospray ionization triple-quadrupole MS. The method involves the use of immunomagnetic beads followed by screening with flow cytometry and UPLC-MS. The high-throughput screening mode is capable of detecting T4 in water at the part-per-trillion level and in the part-per-billion level in urine.

Relevant Phase II enzymes that are responsible for TH metabolism include UGT1A1, UGT1A6 and SULT2A1 while relevant cellular import/export transport proteins include MCT8, OATP1A4 and MRP2. All contribute towards systemic clearance of TH and conjugates from serum whether increasing biliary excretion or moving TH into tissues and across the placenta and BBB. Enzyme induction can only be measured via in vitro cell-based assays and since these enzymes are all controlled by specific nuclear receptors, assays targeting these receptors might act as surrogate measurement (Murk et al 2013). Several methods measuring expression of UGT or SULT mRNA have been published; however, there have been limited efforts to develop higher-throughput methods. The EPA ToxCast Phase I efforts used quantitative nuclease protection assays (qNPA) to screen several hundred chemicals for UGT1A1 and SULT2A1 (Rotroff et al 2010, Sinz et al 2006).

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Event: 959: Increased, Free serum thyroxine (T4)

Short Name: Increased, Free serum thyroxine (T4)

Key Event Component

Process	Object	Action
	thyroxine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	KeyEvent
Aop:367 - Competitive binding to thyroid hormone carrier protein thyroid binding globulin (TBG) leading to altered amphibian metamorphosis	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

blood

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
African clawed frog	Xenopus laevis		NCBI

Data in humans focusing on the thyroid disruption potential of hydroxylated PCBs and PBDEs is scarce, the data are conflicting and suffer from differing analytical and reporting methods.

Hagmar et al 2001a and 2001b examined 4-OH-CB107 and 4-OH-CB187 in adult females and male fishermen from the Baltic Sea and found no associations. Similarly, Bloom et al 2009 found no associations between PFOS and TH in a small study of New York anglers.

Athanasiadou et al 2008 assessed PBDEs in pooled serum samples from 11-15 year old children living near an urban municipal waste site as well as mothers who consume fish from a rural location in Nicaragua. BDE-47 was the most abundant congener found in samples, followed by BDEs 99, 100 and 153. This study was the first to confirm that hydroxylated metabolites (OH-PBDEs) accumulate in human serum, identifying 19 OH-PBDEs – at least six (6) of which were also found and retained in rat serum following exposure to an artificial PBDE mixture (Malmberg et al 2005). The dominant congeners were 4-OH-BDE17 and 4'-OH-BDE-49. These data support the concept that residential exposure to PBDEs is strongly influenced by inhalation and ingestion of house dust rather than consumption of contaminated food.

Dalliare et al 2009 looked at the relationship between TH status and exposure to PCB-153, pentachlorophenol, hexachlorobenzene and hydroxylated PCBs in pregnant Inuit women and their infants. PCB-153 was the most predominant congener found to be elevated most in pregnant women, followed by infant and cord plasma levels. OH-PCB results were a sum of 11 major congeners and found to be higher in pregnant women than cord blood, but highly intercorrelated. Overall, the results suggest that the compounds measured in serum were not significant predictors of TH or TSH concentrations in this population. The strongest results were found for PCP, which was negatively associated with free T4 in neonate cord blood, suggesting PCP reduces the transfer of T4 across the placenta.

Dallaire et al 2009b examined the relationship between TH status and TBG and exposure to 41 different contaminants, including PCBs and metabolites, PBDEs, PFOS, organochlorine pesticides and dioxin-like compounds, in over 500 Inuit adults. Negative associations were reported between rT3 and 14 PCBs, 7 hydroxylated metabolites, methylsulfonyl metabolites and 2 pesticides. Negative associations were also reported between free T4 and hexachorobenzene as TBG concentrations were inversely related to 8 PCBs, 5 hydroxylated metabolites and three pesticides. This was the first large study to examine the effect of PFOS on TH homeostasis in exposed human adults, observing a significant negative associations with TSH, total T3 and TBG while observing a positive association with free T4.

Chevrier et al 2010 measured concentrations of 10 PBDE congeners and TH in 270 pregnant Californian (CHAMACOS cohort) women during the 27th week of gestation. This study reported significant inverse associations between TSH and serum concentrations of BDEs 28, 47, 99, 100 and 153 but did not observe an association between PBDEs and free T4.

Eskenazi et al 2013 measured PBDEs in maternal prenatal and child serum samples and examined the association between blood concentration and attention, motor functioning and cognition at ages 5 and 7 in an ongoing large California cohort (CHAMACOS). They observed weak correlations between cognition, motor function and attention and PBDE concentration in maternal prenatal and child blood at age 7 and the authors claim it largely supporting previous findings in smaller cohorts exposed to both PDBEs and PCBs.

Eguchi et al 2015 measured PCBS, OH-PCBs, PBDEs, methoxylated PBDEs, OH-PBDEs and bromophenols and TH in the serum of Vietnamese cohort composed of human donors from an e-waste recycling site and a rural site. In general, PCBs, OH-PCBs, PBDEs and bromophenols were higher in sera from the recycling site; however, the concentrations of methoxylated PBDEs were higher at the rural site. Positive associations between PCBs and OH-PCBs concentrations and total and free T4 and T3, as well as a negative association with TSH, among females.

Dirinck et al 2016 examined the relationship between PCBs (n=29) and serum hydroxylated PCBs (n=18) and clinically available

markers of thyroid function (TSH, free T4) in 180 subjects recruited upon visitation to the Antwerp University Hospital Department of Endocrinology from 2009 to 2012. The combined regression model for both PCBs and hydroxylates identified PCBs 95 and 99 and 3-OH-CB180 as significant predictors of free T4 , while the model run for just serum hydroxylate identified 3-OH-CB118 and 3-OH-CB180 as major predictors of free T4. The former is a product of metabolizing PCBs 107, 118 and 126 while the latter comes from PCBs 172 and 180.

Key Event Description

Following displacement of T4 from its binding site on TTR by some competitive ligand (like EMD 21388, etc.), the T4 joins the small pool of free hormone found in serum. This increases the amount of free T4 and has been demonstrated in animal models following administration of a xenobiotic competitive ligand.

Kohrle et al (1989) administered 2 umol of EMD 21388 per 100g BW in a single ip injection to euthyroid adult male Sprague-Dawley rats. Rat serum was analyzed for T3 and T4 content via species-adapted RIA and percent free TH was determined via equilibrium dialysis. Serum T4 decreased significantly following 1 hr or administration and remained low for several hours, while % free T4 increased significantly at 1 hr and remained elevated. {insert Figure 5} Previously, both in vitro and in vivo electrophoretic data showed complete inhibition of radiolabeled T4 binding to TTR. Administration of EMD 21388 to rats did not impact T3 concentrations or deiodinase activity.

Lueprasitsakul et al (1990) also administered EMD 21388 to euthyroid adult male Sprague-Dawley rats as a single 2 umol ip injection with additional time points as well as a single injection of 0.3 umol. In addition, one treatment group were exposed to varying doses from 0.2 to 2 umol EDM21388 per 100 g BW. Significant decreases in radiolabeled T4 bound to TTR were found within 3 minutes, reaching a maximum at 10 minutes. A simultaneous increase in % free T4 was noted at 3 minutes and reached a maximum at 10 minutes. {insert Figure 3} These effects were observed for both the high dose of 2 umol and the low dose of 0.3 umol; however, it was noted that % T4 bound to TTR recovered to almost control levels after 3 hours at the low dose. The authors concluded that EMD 21388 administration increased both the free T4 concentration as well as the albumin-bound T4 (which is available in serum and can play a greater role in transport when needed).

Mendel et al (1992) performed additional kinetic studies with radiolabeled T4 and albumin using Sprague-Dawley rats receiving a single ip injection of 2 umol EMD 21388. To overcome the dilution effect found with equilibrium dialysis, ultrafiltration of undiluted serum was employed to measure the % free T4. The % free T4 increased significantly at 20 minutes and the compensatory response of albumin appears to have been saturated after 20 minutes, as shown by the plasma disappearance curve for radiolabeled albumin. {insert Figures 2 and 3} The authors concluded that these data did not confirm TTR is a major carrier of T4 from plasma to liver and other tissues; however, these data also did not distinguish between whether transfer in vivo could be via albumin or from the free pool of T4 in serum.

Chanoine et al (1992) administered low (0.3 umol) and high dose (2 umol) EMD 21388 to Sprague-Dawley rats via single ip injection and a second treatment group had radiolabeled T4 injected 15 minutes following the EMD 21388 adminisatration. Both doses produced a similar significant increase to free T4 in serum within 15 minuteas of administration. {insert Figure 1} Binding of T4 to albumin in serum increased an order of magnitude in both high and low dose treatments. The low dose had no effect on the %T4 bound to TTR in the choroid plexus or the cerebrospinal fluid; however, the high dose did significantly decrease this. {insert Figure 2}

Pedraza et al (1996)

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Total T4 is most often measured using a serum-based diagnostic kit; however, free T4 is considered a more reliable measure of thyroid dysfunction and the only direct measurements for unbound thyroid hormone are equilibrium dialysis and ultrafiltration (Zoeller et al 2007). Large volumes of serum must be used to capture the very low concentrations of free THs and this requires pooling in non-adult animals.

Total T4 is most often measured using human serum based diagnostic kits, but free T4 (and T3) is only directly measured through equilibrium dialysis and ultrafiltration (Midgley 2001). Large volumes of serum must be used due to the very low concentrations of free T4 normally found (0.1% of total T4), which requires pooling of samples taken from fetus or pup. Some researchers have tried to “micronize” this process through combining RIA to measure total TH and dialysis to estimate the free fraction (Zoeller et al 2007). Extracted materials can also be quantified by HPLC. The reference range for free T4 is 9.8 to 18.8 pM/L (Dirinck et al 2016).

T3 is found in similar plasma concentrations to T4 (i.e. 5-10 pM) with < 0.4% being in the unbound state. Measuring free serum T3 is labor intensive and requires equipment not available in many clinical reference laboratories and thus ultrafiltration is often used (Abdalla and Bianco 2014). Immunoassays and MS/MS are also used.

Measuring displacement of T4 from serum transport proteins is done mainly via one of three *in vitro* methods: radioligand binding assay, plasmon resonance-based biosensor, or fluorescence displacement.

Radioligand binding assays, using [^{125}I]-T4 as a label, were developed to demonstrate affinity for xenobiotics to human or rat TTR and TBG (Brouwer and van den Berg 1986, Lans et al 1994). The most commonly used method was first published by Somack et al 1982 and adapted by Hamers et al 2006, Lans et al 1993 and Ucan-Marin et al 2010. Similar assays have been developed using [^{125}I]-T3 as a label for affinity to chicken and bullfrog TTR (Yamauchi et al 2003). Radioligand methods suffer from having to use heavily regulated isotopes and lower throughput to provide free T4 measurements (due to the extra wash/separation procedure needed). The most well-known protocol uses TTR purified from human serum (which may not be as stable as recombinant) and performed in a pure aqueous solution, which may not be as stable for lipophilic compounds (Chauhan et al 2000 is an example using PCBs).

Purkey et al 2001 published a binding assay using polyclonal TTR antibodies covalently bound to sepharose resin which is then mixed with plasma pre-treated with compound of interest, washed and analyzed via HPLC.

Marchesini et al 2006 reported on the development of two surface plasmon resonance(SPR)-based biosensor assays using recombinant TTR and TBG, validated with known thyroid disruptors and structurally related compounds including halogenated phenols, polychlorinated biphenyls, bisphenols and a hydroxylated PCB metabolite (4-OH-CB 14). TH is covalently bound to a gold-layered chip and a mixture of the compound of interest and transport protein are injected in a flow cell passing over the bound TH. The authors found that these biosensor methods were more sensitive (IC50 of 8.6 ± 0.7 nM for rTTR), easier to perform and more rapid that radioligand binding assays and immunoprecipitation-HPLC.

Marchesini et al 2008 applied their biosensor-based screen to 62 chemicals of public health concern and found that hydroxylated metabolites of PCBs (particularly para-hydroxylated ones) and PBDEs (BDEs 47, 49 and 99) displayed the most potent binding to TBG and TTR, confirming many other previous studies. The authors conclude their optimized assays are suitable for high-throughput screening for potential thyroid disruption.

Cao et al 2010, Cao et al 2011 and Ren and Guo 2012 developed the FLU-TTR, based on a protein-binding fluorescent probe (ANSA, or 8-anilo-1-naphthalenesulfonic acid ammonium salt) that becomes highly fluorescent after binding to T4. When the compound of interest is introduced and displaces the ANSA-thyroxine probe, this fluorescence is reduced. This allows generation of binding constant (K) data as opposed to past efforts that generated IC_{50} values. Cao et al 2011 developed a fluorescent microtiter method for pTTR and TBG tested with bisphenol A.

Montano et al 2012 developed a competitive T4-TTR fluorescence displacement assay in a 96-well format, modified from the original method (Nilsson and Petersen 1975) and using a new selective method to extract hydroxylated metabolites while reducing fatty acid interference (modified from Hovander et al 2000).

Aqai et al 2012 described a rapid and isotope-free ($^{13}C_6$-T4) screening of thyroid transport protein ligands, using a competitive binding assay for rTTR using fast ultrahigh performance LC-electrospray ionization triple-quadrupole MS. The method involves the use of immunomagnetic beads followed by screening with flow cytometry and UPLC-MS. The high-throughput screening mode is capable of detecting T4 in water at the part-per-trillion level and in the part-per-billion level in urine.

Relevant Phase II enzymes that are responsible for TH metabolism include UGT1A1, UGT1A6 and SULT2A1 while relevant cellular import/export transport proteins include MCT8, OATP1A4 and MRP2. All contribute towards systemic clearance of TH and conjugates from serum whether increasing biliary excretion or moving TH into tissues and across the placenta and BBB. Enzyme induction can only be measured via in vitro cell-based assays and since these enzymes are all controlled by specific nuclear receptors, assays targeting these receptors might act as surrogate measurement (Murk et al 2013). Several methods measuring expression of UGT or SULT mRNA have been published; however, there have been limited efforts to develop higher-throughput methods. The EPA ToxCast Phase I efforts used quantitative nuclease protection assays (qNPA) to screen several hundred chemicals for UGT1A1 and SULT2A1 (Rotroff et al 2010, Sinz et al 2006).

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Event: 960: Increased, Uptake of thyroxine into tissue

Short Name: Increased, Uptake of thyroxine into tissue

Key Event Component

Process	Object	Action
transport	thyroxine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Key Event Description

T4 (and T3) is actively transported across the cell membrane into target tissues through the action of specific carrier-mediated uptake (simple diffusion probably plays a minor role), where it is T3 that binds to and triggers the nuclear receptors in the target cells (Yen 2001, Zoeller et al 2007). The T3 supply is met via secretion from the thyroid (20%) and through conversion of T4 into T3 (80%) through the action of outer-ring deiodinase enzymes D1 and D3 (Chopra 1996 from Zoeller et al 2007). THs are cleared from serum by the liver following sulfation (via sulfotransferase enzymes) or glucuronidation (via UDP-glucuronosyl transferase enzymes) and ultimately, eliminated in the bile (Hood and Klaasen 2000).

Two major groups of transporters have been identified: organic anionic transport proteins (OATPs) and amino acid transporters (L- and T-type). Several of these transporters have displayed greater affinity and selectivity for T4 and T3 and specific compounds (such as polychlorinated biphenyls and polybrominated diethyl ethers) have been found to bind to the transport proteins either in serum or in various cellular compartments (Zoeller et al 2007)

OATPs transport both iodothyronines as well as sulfated conjugates and the gene family (SLCO) coding for this family of homologous proteins is clustered on human 12p12 (Hagenbuch and Meier 2004). OATP1A2 is expressed in brain, liver and kidney while OATP1B1, -1B2, and -1B3 are expressed in the liver and display high affinity for both T4 and T3 (Friesma et al 2005). OATP1C1 shows binding preference for T4 over T3 and is almost exclusively expressed in brain capillaries, where it is thought to play a role for transport of T4 across the blood brain barrier (Tohyama et al 2004).

There is also evidence that L-type or T-type amino acid transport proteins also play a role in cellular uptake of thyroid hormones. The former transport large neutral branched-chain and aromatic amino acids while the latter are specific to the aromatic amino acids Phe, Tyr and Trp (Visser 2010). The T-type amino acid transport protein TAT1 has been cloned from both rats and humans, is encoded by SLC16A10, and is a member of the monocarboxylate transporter (MCT) family (MCT10)(Kim et al 2002). Both MCT and MCT8 share a high degree of homology and are both highly effective iodothyronine transporters. MCT8 is highly selective for T4 and T, responsible for transporting T3 into neuronal cells and interferes with brain development if absent (Friesma et al 2003, 2006 and 2008). MCT8 is expressed in multiple tissues, including liver, kidney, heart, brain, placenta, thyroid, skeletal muscle and adrenal gland, while MCT10 is also expressed in various tissues, with high expression in muscle, intestine, kidney and pancreas and the former is known to only transport iodothyronine molecules while the latter can also carry aromatic amino acids (Nishimura et al 2008). In terms of transport efficacy, MCT10 appears to be superior to MCT8 for moving T3; however, the reverse is true for T4.

Uptake into hepatocytes is probably mediated through multiple low-affinity/high-capacity and high-affinity/low-capacity processes that can be inhibited by certain molecules, such as fatty acids, bilirubin and indoxyl sulfate (Henneman et al 2001). Km values for these processes are in the micro- to nanomolar range, while the serum concentrations of free T4 and T3 are in the picomolar range.

Accumulation in tissue as well as transport across the placental and blood-brain barrier is dependent on expression level of the various TH membrane transporters, such as those in the MCT and OATP families. MCT8, MCT10, L-type amino acid transporters LAT1 and LAT2, OATP1A2 and OATP4A1 are responsible for transplacental transfer of TH (Loubiere et al 2010), while MCT8 and OATP1C1 are important to transfer across the blood-brain barrier (Roberts et al 2008).

OATP1B1 and OATP1B3 show preference for sulfated THs and are expressed only in the liver

OATP1C1 shows high preference for T4 and almost exclusively expressed in brain capillaries and choroid plexus (Hagenbuch 2007)

LAT1 and LAT2 facilitate bidirectional transport of both T4 and T3 (and aliphatic and aromatic amino acids) across the plasma membrane

MCT8 only transports THs and expressed in choroid plexus, MCT10 also transports aromatic amino acids

Schroder van der Elst et al 1997 injected female rats with synthetic flavonoid [^{125}I]-EMD 49209 and [^{131}I]-T4 (or rats pretreated with EMD 21388), noting rapid clearance of [^{125}I] from serum and rapid uptake of [^{131}I] into tissues. These results show that the

flavonoid itself does not cross the blood-brain barrier, despite the fact that they demonstrate displacement of T4 from TTR and temporarily increase the pool of available free T4.

Schroder van der Elst et al 1998 injected pregnant rats at GD 20 with [^{125}I]-EMD 49209 and observed distribution in maternal tissues, intestinal contents and fetal tissues. No flavonoid was detected in the brain but it was found in all fetal tissues examined, including brain.

TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER

Active transport is required for uptake of T3 and T4 across cell membranes (Heuer 2007). Accumulation in tissue as well as transport across the placental and blood-brain barrier (BBB) is dependent on expression level of the various TH membrane transporters, such as those in the MCT and OATP families. MCT8, MCT10, L-type amino acid transporters LAT1 and LAT2, OATP1A2 and OATP4A1 are responsible for transplacental transfer of TH (Loubiere et al 2010), while MCT8 and OATP1C1 are important to transfer across the blood-brain barrier (Roberts et al 2008). Visser et al 2008 proposed that OATP14 is mainly responsible for moving T4 into the brain/CSF, where it is converted into T3 locally and transported into neurons via MCT8.

Schreiber et al 1995; Schreiber 2002

Roberts et al 2008 examined the expression of MCT8 and OATP14 in male Sprague Dawley rats, male CD-1 mice and human brain tissue via qPCR and immunofluorescent staining as well staining and confocal microscopy in isolated cerebral microvessels and choroid plexus (CP) epithelium. They observed that the main transporter at the BBB is OATP14 whereas MCT8 mediates TH uptake into neuronal cells. MCT8 mRNA and proteins were expressed in cerebral microvessels in all species; however, OATP14 mRNA and protein was only enriched in mouse and rat microvessels. In all species, MCT8 is concentrated on the epithelial cell apical surface and OATP14 primarily on the basal-lateral surface of the CP epithelial cells. These data suggested MCT8 plays a role in TH transport across the BBB and this is supported by the pattern of localization of the two transporters.

Kim et al 2015 screened protein transporters in rat serum for the potential to guide nanoparticles across the BBB (via receptor-mediated transcytosis, or RMT) using an *in vitro* transcytosis assay using rat and human brain microvascular endothelial cells. Eleven (11) proteins were identified as showing potential to penetrate the endothelial cell layer via RMT, including Ttr. Ttr was then incorporated into a quantum dot nanoparticle, administered to male Sprague-Dawley rats via IV and found to cross the BBB in rats via transcytosis, confirmed by *in vivo* imaging, TEM, ICP-MS and confocal microscopy.

TRANSPORT ACROSS PLACENTA

There is a direct role for maternal TH in the development of the fetal CNS starting with the 1st trimester and this maternal TH must be provided to the fetus via transplacental delivery (Chan et al 2002; de Escobar et al 2004). The placenta responds to TH with both the villous and extravillous trophoblasts (EVTs) expressing specific nuclear receptor isoforms for T3. The primary barrier of cells for maternal-fetal exchange are the syncytiotrophoblasts of placental villi, which are in direct contact with maternal blood, and the cytotrophoblasts, which form an additional inner layer of cells (Benirschke et al 2000). Free T4 is the believed to be the primary TH transported across the placenta and fetal free T4 levels reach ~ 40-50% of maternal concentrations by the early 2nd trimester and peak in the early 3rd trimester, where they remain at levels higher than the corresponding maternal concentration (Calvo et al 2002; Hume et al 2004).

Accumulation in tissue as well as transport across the placental and blood-brain barrier is dependent on expression level of the various TH membrane transporters, such as those in the MCT and OATP families. MCT8, MCT10, L-type amino acid transporters LAT1 and LAT2, OATP1A2 and OATP4A1 are responsible for transplacental transfer of TH (Loubiere et al 2010), while MCT8 and OATP1C1 are important to transfer across the blood-brain barrier (Roberts et al 2008). The MCT8, OATP4A1 and LAT1 are localized at the apical membrane of the syncytiotrophoblasts while MCT10 is localized in the cytotrophoblasts during the 1st trimester (Ritchie and Taylor 2001; Sato et al 2003; Chan et al 2006; Loubiere et al 2010)

Displacement of T4 from transport proteins during the developmental stage could have consequences for both fetal development and later in adulthood (Morse et al 1996). Transfer of maternal TH across the placenta is essential to neurodevelopment and even temporary disruption during the perinatal period can have long-term adverse health effects (Zoeller and Rovet 2004). Animal studies have confirmed that perinatal exposure to PBDEs can adversely affect neurodevelopment of CNS; however, the mechanisms remain elusive and the evidence in humans that hydroxylated metabolites of PBDEs is equivocal (Costa et al 2014).

TTR mediates transport through the placenta and the hydroxylated metabolites of PCBs and PBDEs have been found to more potent than the natural ligand T4 and thus, competitive binders. The presence of these compounds in maternal and infant blood have been associated with changes in TH, developmental endpoints and fertility in humans (Chevrier et al 2010, Harley et al 2010, Koopman-Esseboom et al 1994). Several studies have demonstrated links between decreased T4 levels and neurodevelopmental and neurobehavioral adverse outcomes in mice exposed specifically to BDE-99 (Branchi et al 2002; Viberg et al 2002).

Darnerud et al 1996 treated pregnant C57BL and NMRI mice on GD 13 with a single gavage at two doses of [^{14}C]-labelled 3,3',4,4'-tetraCB (PCB 77) and experiment was terminated after 4 days, measuring radioactivity and TH in maternal and fetal liver and plasma. Competitive binding assay was also done with [^{125}I]-T4 complex from samples of fetal and maternal plasma. Dose-dependent uptake of [^{14}C] were noted in both maternal and fetal plasma and liver, with fetal plasma radioactivity levels being 4- to 9-fold higher than maternal levels and corresponded to a single metabolite (4-OH-tetraCB). Gel electrophoresis confirmed the [14C] was bound to fetal serum TTR and the fetal sera samples at the top dose (10 mg/kg) showed 50% TTR binding relative to controls, along with significant decrease in free and total serum T4.

Morse et al 1996 (also see Morse et al 1993) treated Wistar rats with Aroclor 1254 at 2 doses via daily oral exposure from GD 10 to 16, with blood and tissue (brain, liver) collected from dams and fetuses on GD 20 with pups reared until PND Day 21 (with blood and tissue collected at PND 4 and 21). The biological samples were analyzed for TH, type II deiodinase activity and levels of PCBs and metabolites. Maternal exposure to Aroclor 1254 significantly reduced both free and total T4 in the serum of fetus and neonate (Day 4) in a dose-dependent manner (but less pronounced at PND 21 and absent at Day 90). At GD 20, levels of T4 in fetal forebrain and cerebellum, and at PND 21 female weanlings at the high dose (25 mg/kg) still had significantly decreased forebrain T4. In the fetus, deiodination (T4 to T3) was significantly increased in forebrain and in the female weanling, only at the low dose was deiodination significantly decreased. Similarly, glucuronidation was significantly decreased in the fetus and significantly increased in the female weanling. Accumulation of mainly one metabolite (2,3,3',4',5-pentachloro-4-biphenylol, or 4-OH-pentaCB; possibly from PCB 118 or PCB 126) was noted in fetal serum and forebrain as well as neonatal and weanling plasma and the concentrations of the metabolite in plasma relative to the more persistent parent congeners (PCB 153) were increased all the way to 90 days (and the plasma levels of the offspring exceeded that of dam all the way to 90 days). These data show that maternal exposure to PCBs can result in accumulation of hydroxylated metabolites in fetal plasma that reduces T4 and, as a result, reduces brain levels of T4 with a compensatory increase in brain deiodination to maintain brain T3 concentration.

Pedraza et al 1996 treated pregnant Wistar rats first with methimazole (to block hormone synthesis) and then continuous infusion of EMD 21388 and T4 from GD 11 to 21, noting decreased total T4, increased free T4 and decreased T3 in maternal serum, increase of T3 in placenta and led to measurable amounts of parent compound in fetal serum along with decreased total T4 and increased T3.

Schroder van der Elst et al 1998 injected pregnant rats at GD 20 with [^{125}I]-EMD 49209 and observed distribution in maternal tissues, intestinal contents and fetal tissues. No flavonoid was detected in the maternal brain but it was found in all fetal tissues examined, including brain. It should be noted though that TTR is the principal carrier in the fetal rat and the EMD flavonoids were designed as T4 analogs and only bind to TTR (and not to albumin or TBG). Shortly after birth, TTR production decreases to nearly zero and thus, interference with TTR-T4 during certain developmental windows might impact availability of thyroid hormone in certain tissues at critical time periods.

Sinjari and Darnerud (1998) injected C57BL mice on GD 16 with 5 doses of [^{14}C]-labelled metabolites of PCB 77 (4-OH-tetraCB, two different 4-OH-pentaCB metabolites of PCB105), sacrificed 24 hours later, plasma and tissues collected from dam and fetus and analyzed for [^{14}C], total T4 and liver microsomes. Partial dose dependency was found for both maternal and fetal decreased total T4 for 4-OH-tetraCB and one of the pentaCBs. Also, placental transfer to fetal plasma was dose dependent and, at lower doses (less than 5 mg/kg), fetal serum levels of 4-OH-tetraCB were 2-fold higher than maternal serum levels. The authors conclude that doses in excess of 5 mg/kg saturate ligand binding, as effects measured at concentrations higher than this are not dose-related (however, there was extensive biliary excretion of 4-OH-tetraCB at the highest doses (20 and 50 µmol/kg). These results suggest that hydroxylated metabolites of PCBs are transferred to fetus upon maternal exposure, but did not induce a CYP1A1 or CYP1A2 response in the dam and competitive binding with T4 may not be the only mechanism behind noted adverse fetal effects from T4 modulation.

Meerts et al 2002 treated pregnant rats with 5 mg/kg of 4-OH-CB107 (radiolabeled and non-labelled) on GD 10 to 16, noting accumulation in the fetal compartment. The complex between TTR and [^{14}C]-4-OH-CB107 was detected in serum in both dam and fetus. Total and free serum T4 were reduced in fetus at GD 17 and 20. T4 concentration in fetal forebrain homogenate was reduced at GD20 and deiodination of T4 to T3 was increased at GD 17. No changes were noted in maternal or fetal hepatic UDP-UGT activity, type 1 deiodination, or EROD activity. These data show that TTR-mediated transport of xenobiotics, like the metabolites of PCB 107, can result in transfer from mother to fetus and can result in reduced fetal T4 (although it should be noted that fetal T3 remained unaffected). Furthermore, there was significant increase of fetal TSH at GD20, indicating stimulation of the HPT axis.

Inoue et al 2004 reported that PFOS can cross the placental barrier in humans

McKinnon et al 2005

Morse et al 2005 injected pregnant Wistar rats on GD 13 with single dose of [^{14}C]-labelled 3,3',4,4'-tetraCB (PCB 77) and tracked metabolism for 7 days. The main metabolite was 4-OH-CB77 was found in maternal liver and plasma, placental tissue and fetal plasma, with 4-OH-CB77 accumulating 100-fold in fetus over the observation period with levels in fetal plasma being 14-fold higher than maternal plasma on GD 20. Similarly, while maternal serum T4 was initially significantly reduced and recovered by GD 20, the fetal plasma T4 was found to be significantly reduced relative to maternal T4 on GD 20. These data show that exposure to PCBs during pregnancy can result on transfer of metabolites to fetus (and these are competitive with T4 at the TTR binding site).

Riu et al 2008 fed pregnant Wistar rats with [14C]-decabromodiphenyl ether (DBDE) over 96 hr of late gestation (GD 16 to 19) and tissues analyzed via HPLC. More than 19% of the administered dose was recovered, with 2/3 of this eliminated via feces, and results in accordance with past findings in Fisher and Sprague Dawley rats. Small amounts were found to cross both the blood-

brain and placental barriers and hydroxylated octaBDE was found in all tissues and fetus.

Dalliare et al 2009a looked at the relationship between TH status TBG and exposure to PCB-153, pentachlorophenol, hexachlorobenzene and hydroxylated PCBs in pregnant Inuit women and their infants. PCB-153 was the most predominant congener found to be elevated most in pregnant women, followed by infant and cord plasma levels. OH-PCB results were a sum of 11 major congeners and found to be higher in pregnant women than cord blood, but highly intercorrelated. Overall, the results suggest that the compounds measured in serum were not significant predictors of TH or TSH concentrations in this population. The strongest results were found for PCP, which was negatively associated with free T4 in neonate cord blood, suggesting PCP reduces the transfer of T4 across the placenta. This confirmed previous findings of Sandau et al 2002, but has conflicted with other study populations and published reports; however, has biological plausibility as PCP has been reported to have a binding affinity twice that of the natural ligand for TTR (van den Berg 1990).

Loubiere et al 2010 described the ontogeny of TH transporters MCT8, MCT10, LAT1, LAT2, OATP1A2 and OATP4A1 in over 100 placenta samples collected across gestation via RNA extraction and qRT-PCR. These mRNA data showed increasing expression of MCT8, MCT10, OATP1A2 and LAT1 throughout gestation, while OATP4A1 and CD98 (associated with LAT activity) mRNA fell to a nadir in the late 1st and early 2nd trimester. Immunohistochemistry data localized MCT10 and OATP1A2 for the first time to EVTs as well as syncytiotrophoblasts.

How it is Measured or Detected

Total T4 is most often measured using human serum based diagnostic kits, but free T4 (and T3) is only directly measured through equilibrium dialysis and ultrafiltration (Midgley 2001). Large volumes of serum must be used due to the very low concentrations of free T4 normally found (0.1% of total T4), which requires pooling of samples taken from fetus or pup. Some researchers have tried to “micronize” this process through combining RIA to measure total TH and dialysis to estimate the free fraction (Zoeller et al 2007). Extracted materials can also be quantified by HPLC. The reference range for free T4 is 9.8 to 18.8 pM/L (Dirinck et al 2016).

T3 is found in similar plasma concentrations to T4 (i.e. 5-10 pM) with < 0.4% being in the unbound state. Measuring free serum T3 is labor intensive and requires equipment not available in many clinical reference laboratories and thus ultrafiltration is often used (Abdalla and Bianco 2014). Immunoassays and MS/MS are also used.

Measuring displacement of T4 from serum transport proteins is done mainly via one of three *in vitro* methods: radioligand binding assay, plasmon resonance-based biosensor, or fluorescence displacement.

Radioligand binding assays, using [^{125}I]-T4 as a label, were developed to demonstrate affinity for xenobiotics to human or rat TTR and TBG (Brouwer and van den Berg 1986, Lans et al 1994). The most commonly used method was first published by Somack et al 1982 and adapted by Hamers et al 2006, Lans et al 1993 and Ucan-Marin et al 2010. Similar assays have been developed using [^{125}I]-T3 as a label for affinity to chicken and bullfrog TTR (Yamauchi et al 2003). Radioligand methods suffer from having to use heavily regulated isotopes and lower throughput to provide free T4 measurements (due to the extra wash/separation procedure needed). The most well-known protocol uses TTR purified from human serum (which may not be as stable as recombinant) and performed in a pure aqueous solution, which may not be as stable for lipophilic compounds (Chauhan et al 2000 is an example using PCBs).

Purkey et al 2001 published a binding assay using polyclonal TTR antibodies covalently bound to sepharose resin which is then mixed with plasma pre-treated with compound of interest, washed and analyzed via HPLC.

Marchesini et al 2006 reported on the development of two surface plasmon resonance(SPR)-based biosensor assays using recombinant TTR and TBG, validated with known thyroid disruptors and structurally related compounds including halogenated phenols, polychlorinated biphenyls, bisphenols and a hydroxylated PCB metabolite (4-OH-CB 14). TH is covalently bound to a gold-layered chip and a mixture of the compound of interest and transport protein are injected in a flow cell passing over the bound TH. The authors found that these biosensor methods were more sensitive (IC50 of 8.6 ± 0.7 nM for rTTR), easier to perform and more rapid that radioligand binding assays and immunoprecipitation-HPLC.

Marchesini et al 2008 applied their biosensor-based screen to 62 chemicals of public health concern and found that hydroxylated metabolites of PCBs (particularly para-hydroxylated ones) and PBDEs (BDEs 47, 49 and 99) displayed the most potent binding to TBG and TTR, confirming many other previous studies. The authors conclude their optimized assays are suitable for high-throughput screening for potential thyroid disruption.

Cao et al 2010, Cao et al 2011 and Ren and Guo 2012 developed the FLU-TTR, based on a protein-binding fluorescent probe (ANSA, or 8-anilo-1-naphthalenesulfonic acid ammonium salt) that becomes highly fluorescent after binding to T4. When the compound of interest is introduced and displaces the ANSA-thyroxine probe, this fluorescence is reduced. This allows generation of binding constant (K) data as opposed to past efforts that generated IC_{50} values. Cao et al 2011 developed a fluorescent microtiter method for pTTR and TBG tested with bisphenol A.

Montano et al 2012 developed a competitive T4-TTR fluorescence displacement assay in a 96-well format, modified from the original method (Nilsson and Petersen 1975) and using a new selective method to extract hydroxylated metabolites while reducing fatty acid interference (modified from Hovander et al 2000).

Aqai et al 2012 described a rapid and isotope-free ($^{13}C_6$-T4) screening of thyroid transport protein ligands, using a competitive

binding assay for rTTR using fast ultrahigh performance LC-electrospray ionization triple-quadrupole MS. The method involves the use of immunomagnetic beads followed by screening with flow cytometry and UPLC-MS. The high-throughput screening mode is capable of detecting T4 in water at the part-per-trillion level and in the part-per-billion level in urine.

Relevant Phase II enzymes that are responsible for TH metabolism include UGT1A1, UGT1A6 and SULT2A1 while relevant cellular import/export transport proteins include MCT8, OATP1A4 and MRP2. All contribute towards systemic clearance of TH and conjugates from serum whether increasing biliary excretion or moving TH into tissues and across the placenta and BBB. Enzyme induction can only be measured via in vitro cell-based assays and since these enzymes are all controlled by specific nuclear receptors, assays targeting these receptors might act as surrogate measurement (Murk et al 2013). Several methods measuring expression of UGT or SULT mRNA have been published; however, there have been limited efforts to develop higher-throughput methods. The EPA ToxCast Phase I efforts used quantitative nuclease protection assays (qNPA) to screen several hundred chemicals for UGT1A1 and SULT2A1 (Rotroff et al 2010, Sinz et al 2006).

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Event: 961: Increased, Clearance of thyroxine from tissues

Short Name: Increased, Clearance of thyroxine from tissues

Key Event Component

Process	Object	Action
metabolic process	thyroxine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

liver

Key Event Description

Thyroxin (T4) and T3 are metabolized and cleared from tissues in a number of ways: inner ring or outer ring deiodination via specific enzymes, conjugation (glucuronidation or sulfation), oxidative deamination and ether-linked cleavage (Zoeller et al 2007).

Deiodination:

There are three types of deiodinase enzymes. D1 and D2 convert T4 to T3 by removing an iodine atom from the outer ring while D3 removes an iodine atom from the inner ring, converting T4 to reverse T3. Differential expression of these enzymes during brain development are critical to the functionality of thyroid hormone in different areas of the fetal brain.

Much of the T4 is carried to the liver, where it is transported across the cellular membrane, converted into T3 via deiodination as mediated by deiodinase enzymes, and it is this T3 that triggers the TH receptors found in the nucleus. Roughly 80% of the T3 needed is produced via outer-ring deiodination of T4, which "activates" T4 to T3 (as opposed to inner-ring deiodination, which "degrades" T4 to reverse T3 which is eliminated). About 30% of the T4 produced daily (~ 130 nmol) is converted to roughly 40 nmol of T3 (Visser 2012) via enzyme D1 (liver, kidney) while conversion to rT3 accounts for roughly 40% of T4 turnover and is mediated via enzyme D3 (brain, placenta, fetus).

Conjugation:

Glucuronidation and sulfation of T4 accounts for the rest of the metabolized T4 and leads to rapid elimination through bile. It is thought that 20% of daily T4 production is eliminated through biliary excretion of glucuronide conjugates. Glucuronidation is carried out by UDP-glucuronoyltransferase (UGT) enzymes (Hood and Klaassen 2000a, 2000b) and appears to be more important in murine species than in man (Henneman and Visser 1997) and sulfation of T4 is done largely through an initial inner ring deiodination step (via D3). Circulating levels of THs in serum can be affected by compounds that induce the activity of UDP-UGT enzymes.

Uptake into the liver involves "high affinity, low capacity" and "low affinity, high capacity" processes with Km values in the nano- to micro-molar range (as opposed to the free T3 and T4 concentrations, which are in the picomolar range) (Henneman et al 2001 from Visser 2010). Both MCT8 and MCT 10 can transport THs; however, MCT8 is expressed in human liver where MCT10 is not and MCT8 display higher efficacy of cellular uptake and efflux relative to T3 (Ref 12 in Visser 2010).

Data in animals for PCBs

Previous reports have been made showing serum TH decreases in rats and mice in response to PCBs, PCB congeners and TCDD and these decreases have been thought to be driven by UDP-UGT (particularly 1A1 and 1A6)(Barter and Klaassen 1994, Schuur et al 1997, Van Birgelen et al 1995, Visser 1996)

Hallgren et al 2001, Hallgren and Darnerud 2002 showed that both T4 and T3 are significantly decreased following exposure to PCB mixtures or individual congeners.

Kato et al 2003 (and Kato et al 2002) showed for the first time that a commercial PCB mixture (Kanechlor-500, KC500) decreased serum TH without an increase in glucuronidation of T4. Male Wistar rats and ddy mice were given a single ip injection of 100 mg/kg and 4 days later, organ weights were measured and microsomal enzymes measured. Significant increases were noted for both endpoints in both species; however, treatment with PCBs led to significant increases in UDP-UGT activity in rats but not mice. Gene expression of UDP-UGTs was also examined and, again, rats (but not mice) displayed time-dependent increases in levels of UGT1A1 and UGT1A6 following treatment with PCBs. This agrees with past reports showing that clofibrate, phenobarbital, pregnenolone-16-alpha-carbonitrile and beta-naphthoflavone decrease serum TH and increase hepatic UDP-UGT activity in rats but not mice (Viollon-Abadie et al 1999).

This implies that mice may reduce serum TH through a mechanism that does not involve increased glucuronidation, but may involve a TTR-associated pathway (as hydroxylated metabolites of PCBs have been displayed high affinity for TTR in *in vitro* studies). Kanechlor-500 does not display any appreciable amount of outer ring deiodination activity (which would convert serum T4 to T3) and treatment with the mixture did not significantly change TSH levels (indicating there is no induction of the thyroid feedback loop from the measured decreases in serum TH).

Kato et al 2004 performed the same experiment next with Wistar and Gunn rats, the latter species being a Wistar mutant strain that lacks UGT1A isoforms. Both species showed serum T4 (free and total) decrease after single injection of either KC500 or pentaCB and only Wistar rats showed an associated increase in UDP-UGT activity. Significant decrease in type I deiodinase was observed in both rats in addition to detection of hydroxylated PCB metabolites bound to TTR. Gunn rats treated with clofibrate also showed decreased serum T4 without an increase in UDP-UGT activity (Visser et al 1993). These results imply that decreases of serum TH by PCB or pentaCB were managed by formation of OH-PCB metabolites that were then transported by TTR. This is supported by the fact that the main metabolite found in KC500-treated rats was 4-OH-2,3,3',4',5-pentachlorobiphenyl, which displays a binding affinity towards TTR that exceeds that of T4 by more than 3-fold (Meerts et al 2002). In fact, the dihydrohxylated PCBs show several fold higher affinity than the monohydroxylated PCBs (Lans et al 1993). It should be noted that an increase in sulfation via SULT enzymes may also offer an esxplanation for the observed results.

Kato et al 2007 treated Wistar and Gunn rats with KC500 at a lower dose (10 mg/kg) once daily for 10 days, noting decrease in total and free serum T4 as well as the differential UDP-UGT response across the different strains. Clearance of [^{125}I]T4 from serum was higher in both species treated with KC500 and accumulation in several tissues, particularly the liver, was observed. These data imply that reduction of serum TH from exposure to KC500 would be mediated through accumulation in the tissues and not through an increase in glucuronidation. In addition, competitive inhibition by PCB or its metabolites with serum transport proteins (like TTR) could also decrease serum T4 by inducing a change in tissue distribution, especially the liver where more than 40% of[^{125}I]T4 accumulated following treatment.

Kato et al 2009 treated C57BL/6 and DBA/2 mice with the heptaCB metabolite 4-OH-CB187, decreasing free and total serum T4 with no observed UDP-UGT activity or effect on TSH. A number of OH-PCBs have been identified in human serum, including 4-OH-CB107, 3-OH-CB153, 4-OH-CB146, 3'-OH-CB138 and 4-OH-CB187 (which specifically has a 5-fold higher affinity for TTR relative to T4)(Hovander et al 2002). Levels of [^{125}I]T4-TTR were decreased with accompanying increases in binding to TBG and albumin in both strains of mice. Finally, T4 levels increased in tissues, particularly the liver and kidney. Decreases in total and free serum T4 mediated by 4-OH-CB187 were observed in wild-type and TTR-heterozygous mice but not in TTR-deficient mice, with heterozygous mice displaying a smaller decrease in T4 relative to TTR-deficient mice. In both strains of mice, treatment with 4-OH-

CB187 promoted clearance of [^{125}I]T4 from serum relative to controls and serum pharmacokinetic data were estimated, along with tissue-to-serum (K_p value) concentration ratios and [^{125}I]T4 tissue distribution levels. These data imply that 4-OH-CB187 inhibits formation of the [^{125}I]T4-TTR complex, which may lead to a change in tissue distribution, with accumulation in the liver and kidney mainly.

Kato et al 2012 treated C57BL/6 (wild type) and TTR-null mice with single ip injections of pentaCB at 112 mg/kg, noting significant decreases in total serum T4 and T4-TTR complex and measuring [^{125}I]T4 clearance from serum and accumulation in tissues. Treatment with pentaCB resulted in decrease of [^{125}I]T4-TTR and increase in [^{125}I]T4-albumin and [^{125}I]T4-TBG complexes in wild type mice, but not in TTR-deficient mice, although liver accumulation was noted in both strains independent of UDP-UGT activity. These data imply that penta-CB mediated increases in T4 liver concentration occurs mainly through inhibition of efflux of T4 and/or promotion on influx of T4 into hepatic cells (which is a receptor mediated process independent of TTR transport at the liver).

Kato et al 2013 treated C57BL/6 and DBA/2 mice with 50 mg/kg CB118 (pentaCB) in a single ip injection for 5 days, noting decreased serum T4 in both strains and decrease in TSH for the DBA/2 mice but not C57BL/6. CB118-mediated changes in [^{125}I]T4 complexes with TBG, albumin and TTR were only observed in C57BL/6 mice (and not DBA/2), despite [^{125}I]T4 accumulation in the liver of both strains. It is thought that the strain differences are dependent on differences in induction of CYP1A enzymes responsible for the hydroxylation of PCBs (creating metabolites that display far greater affinity for TTR than the natural T4 ligand).

Martin and Klaassen 2010 treated male Sprague Dawley rats with Aroclors 1242 and 1254; PCBs 95, 99, 118, 126 or TCDD at 4 doses via gavage daily for 7 days, then measured serum TH via radioimmunoassay and induction of hepatic Cyp1a and Cyp2b. This study was the first to examine all three classes of PCB congeners: TCDD-type (no chlorine substitutions in ortho position, high affinity for arylhydrocarbon receptor, induce Cyp1a, PCBs 77 and 126), PB-type (at least 2 ortho substitutions, low affinity for AhR, induce Cyp2b, PCBs 28, 95, 99, 101 and 153) or mixed type (1 ortho substitution, low affinity for AhR, induce both Cyp1a and Cyp2b, Aroclors and PCB 118). This study showed that PB-type and mixed type PCB congeners are more effective than TCDD type in reducing serum T4, with Aroclor 1254 (mixed) and PCBs 99 (PB) and 118 (mixed) producing the greatest reduction in serum T4 (as well as T3). Serum TSH was not affected by any compound. Total and free serum T4 was decreased by all treatments in a dose-dependent manner; however marked reduction were noted following treatment with Aroclor 1254, PCB 99 and PCB 118. PCB 118 and 126 caused significant increase in Cyp1a activity while Aroclor 1254 and PCBs 99 and 118 significantly induced Cyp2b. Thus, it appears TCDD type congeners induce CYP1A2 (EROD) activity and UGT-UDP activity in the liver (associated wth binding at AhR) while PB type congeners induce CYP1B2 (PROD) activity and do not induce UGT-UDPs in the liver (associated with increased tissue uptake).

The PB type congeners may induce Oatp1a4 activity to increase clearance from plasma and enhance tissue uptake. Guo et al 2002 reported increase of Oatp1a4 following treatment with PCB 99 (and a decrease following treatment with PCB 126, a TCDD type congener). There are also reports of PB type congeners that accumulate in the liver with little to no increase in glucuronidation or biliary excretion and no changes in serum binding proteins, such as PCB 153, which implies a possible induction of OATP hepatic cellular transport proteins (Kato et al 2011).

Martin et al 2012 treated male Wistar rats with Aroclors 1242 and 1254, PCBs 95, 99, 118 and 126 and TCDD via gavage one per day for 7 days, followed 24 hours later with injection of [^{125}I]T4 and collection of urine, blood, bile and urine. No treatments increased urinary excretion of [^{125}I]T4, but serum T4 was reduced in all treatments and biliary excretion increased following treatment of Aroclor 1254, PCBs 118 and 126, and TCDD as measured by induction of UDP-UGT activity in the liver. PCBs 95 and 99 (PB type congeners) did not induce UGT-UDP activity despite very large and rapid decrease of serum [^{125}I]T4 by PCB 99. These data imply that increased tissue uptake (perhaps through increased TH transport across cell membranes) is another mechanism by which serum T4 can be reduced.

Kato et al 2013 showed that PCB 118 (mixed type) mediated changes in tissue distribution and transport proteins in C57BL/6 mice, but not DBA/2 mice. Kato et al 2012 showed the same with synthesized 2,2',4,5,5'-pentaCB (PCB 101, PB type). Kato et al 2014 showed that PCB 77 (TCDD type) mediated changes in tissue distribution and transport proteins in DBA/2 mice, but not C57BL/6 mice.

Erratico et al 2012 used pooled and single-donor human liver microsomes, human recombinant cytochrome P450 (CYP) enzymes and CYP-specific antibodies to evaluate the oxidative metabolism of BDE-99. Ten (10) hydroxylated metabolites were produced by human microsomes and identified via HPLC-MS/MS and rates of formation were determined, including several that are much more potent than the natural ligand. All ten were found to be catalyzed solely by CYP2B6. Previous studies had also shown formation of hydroxylated metabolites of BDE-99 by human hepatic preparations (Lupton et al 2009, 2010; Stapleton et al 2009); however, fewer OH-PBDEs and additional CYP enzymes were found in similar work done with rat microsomes (Erratico et al 2011).

Feo et al 2013 incubated BDE-47 and recombinant CYPs, measuring the metabolites via GC-MS/MS, as well as specific kinetic studies with BDE-47, CYP2B6 and pooled human liver microsomes. Six (6) OH-PBDEs were found to be catalyzed by CYP2B6 and additional metabolites were identified upon GC-MS/MS (including the novel finding of dihydroxylated metabolites) and these metabolites have been previously found in human serum (Athanasiadou et al 2008; Qui et al 2009). The kinetic studies showed that hydroxylation can occur at low concentrations and that CYPT2B6 has high affinity for BDE-47. CYP2C19 and CYP3A4 were also suggested to play minor roles in the formation of OH-PBDEs.

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Thyroid hormone uptake into human tissues has been measured by analyzing the rate of disappearance of radiolabeled TH from plasma into rapidly and slowly equilibrating tissue compartments (Visser 2010).

Measuring the rate of T4 glucuronidation and sulfation as well as biliary excretion informs the mechanism of action of thyroid system modulation. Studies involving knock/out mice and thyroidectomized rats also inform this mechanism.

Total T4 is most often measured using human serum based diagnostic kits, but free T4 (and T3) is only directly measured through equilibrium dialysis and ultrafiltration (Midgley 2001). Large volumes of serum must be used due to the very low concentrations of free T4 normally found (0.1% of total T4), which requires pooling of samples taken from fetus or pup. Some researchers have tried to “micronize” this process through combining RIA to measure total TH and dialysis to estimate the free fraction (Zoeller et al 2007). Extracted materials can also be quantified by HPLC. The reference range for free T4 is 9.8 to 18.8 pM/L (Dirinck et al 2016).

T3 is found in similar plasma concentrations to T4 (i.e. 5-10 pM) with < 0.4% being in the unbound state. Measuring free serum T3 is labor intensive and requires equipment not available in many clinical reference laboratories and thus ultrafiltration is often used (Abdalla and Bianco 2014). Immunoassays and MS/MS are also used.

Measuring displacement of T4 from serum transport proteins is done mainly via one of three *in vitro* methods: radioligand binding assay, plasmon resonance-based biosensor, or fluorescence displacement.

Radioligand binding assays, using [^{125}I]-T4 as a label, were developed to demonstrate affinity for xenobiotics to human or rat TTR and TBG (Brouwer and van den Berg 1986, Lans et al 1994). The most commonly used method was first published by Somack et al 1982 and adapted by Hamers et al 2006, Lans et al 1993 and Ucan-Marin et al 2010. Similar assays have been developed using [^{125}I]-T3 as a label for affinity to chicken and bullfrog TTR (Yamauchi et al 2003). Radioligand methods suffer from having to use heavily regulated isotopes and lower throughput to provide free T4 measurements (due to the extra wash/separation procedure needed). The most well-known protocol uses TTR purified from human serum (which may not be as stable as recombinant) and performed in a pure aqueous solution, which may not be as stable for lipophilic compounds (Chauhan et al 2000 is an example using PCBs).

Purkey et al 2001 published a binding assay using polyclonal TTR antibodies covalently bound to sepharose resin which is then mixed with plasma pre-treated with compound of interest, washed and analyzed via HPLC.

Marchesini et al 2006 reported on the development of two surface plasmon resonance(SPR)-based biosensor assays using recombinant TTR and TBG, validated with known thyroid disruptors and structurally related compounds including halogenated phenols, polychlorinated biphenyls, bisphenols and a hydroxylated PCB metabolite (4-OH-CB 14). TH is covalently bound to a gold-layered chip and a mixture of the compound of interest and transport protein are injected in a flow cell passing over the bound TH. The authors found that these biosensor methods were more sensitive (IC50 of 8.6 ± 0.7 nM for rTTR), easier to perform and more rapid that radioligand binding assays and immunoprecipitation-HPLC.

Marchesini et al 2008 applied their biosensor-based screen to 62 chemicals of public health concern and found that hydroxylated metabolites of PCBs (particularly para-hydroxylated ones) and PBDEs (BDEs 47, 49 and 99) displayed the most potent binding to TBG and TTR, confirming many other previous studies. The authors conclude their optimized assays are suitable for high-throughput screening for potential thyroid disruption.

Cao et al 2010, Cao et al 2011 and Ren and Guo 2012 developed the FLU-TTR, based on a protein-binding fluorescent probe (ANSA, or 8-anilo-1-naphthalenesulfonic acid ammonium salt) that becomes highly fluorescent after binding to T4. When the compound of interest is introduced and displaces the ANSA-thyroxine probe, this fluorescence is reduced. This allows generation of binding constant (K) data as opposed to past efforts that generated IC_{50} values. Cao et al 2011 developed a fluorescent microtiter method for pTTR and TBG tested with bisphenol A.

Montano et al 2012 developed a competitive T4-TTR fluorescence displacement assay in a 96-well format, modified from the original method (Nilsson and Petersen 1975) and using a new selective method to extract hydroxylated metabolites while reducing fatty acid interference (modified from Hovander et al 2000).

Aqai et al 2012 described a rapid and isotope-free ($^{13}C_6$-T4) screening of thyroid transport protein ligands, using a competitive binding assay for rTTR using fast ultrahigh performance LC-electrospray ionization triple-quadrupole MS. The method involves the use of immunomagnetic beads followed by screening with flow cytometry and UPLC-MS. The high-throughput screening mode is capable of detecting T4 in water at the part-per-trillion level and in the part-per-billion level in urine.

Relevant Phase II enzymes that are responsible for TH metabolism include UGT1A1, UGT1A6 and SULT2A1 while relevant cellular import/export transport proteins include MCT8, OATP1A4 and MRP2. All contribute towards systemic clearance of TH and

conjugates from serum whether increasing biliary excretion or moving TH into tissues and across the placenta and BBB. Enzyme induction can only be measured via in vitro cell-based assays and since these enzymes are all controlled by specific nuclear receptors, assays targeting these receptors might act as surrogate measurement (Murk et al 2013). Several methods measuring expression of UGT or SULT mRNA have been published; however, there have been limited efforts to develop higher-throughput methods. The EPA ToxCast Phase I efforts used quantitative nuclease protection assays (qNPA) to screen several hundred chemicals for UGT1A1 and SULT2A1 (Rotroff et al 2010, Sinz et al 2006).

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

Short Name: T4 in serum, Decreased

Key Event Component

Process	Object	Action
abnormal circulating thyroxine level	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:194 - Hepatic nuclear receptor activation leading to altered amphibian metamorphosis	KeyEvent
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	KeyEvent
Aop:367 - Competitive binding to thyroid hormone carrier protein thyroid binding globulin (TBG) leading to altered amphibian metamorphosis	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered eye structure	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term

serum

Evidence for Perturbation by Stressor

Propylthiouracil

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

Perchlorate

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

Methimazole

Classic positive control

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

The overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in zebrafish (Thienpont et al., 2011), amphibian and lamprey metamorphoses is well established (Manzon and Youson, 1997; Yaoita and Brown, 1990; Furlow and Neff, 2006). Their existence and importance has also been described in many different animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species depends on the expression and function of specific proteins (e.g receptors or enzymes) under TH control and may vary across species and tissues. As such extrapolation regarding TH action across species should be done with caution.

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

Key Event Description

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

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

Normal serum T4 reference ranges can be species and lifestage specific. In rodents, serum THs are low in the fetal circulation, increasing as the fetal thyroid gland becomes functional on gestational day 17, just a few days prior to birth. After birth serum hormones increase steadily, peaking at two weeks, and falling slightly to adult levels by postnatal day 21 (Walker et al., 1980; Harris et al., 1978; Goldey et al., 1995; Lau et al., 2003). Similarly, in humans, adult reference ranges for THs do not reflect the normal ranges for children at different developmental stages, with TH concentrations highest in infants, still increased in childhood, prior to a decline to adult levels coincident with pubertal development (Corcoran et al. 1977; Kapelari et al., 2008). In some frog species, there is an analogous peak in thyroid hormones in tadpoles that starts around embryonic NF stage 56, peaks at Stage 62 and the declines to lower levels by Stage 56 (Sternberg et al., 2011; Leloup and Buscaglia, 1977).

How it is Measured or Detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is the radioimmunoassay (RIA). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is a commonly used as a human clinical test method. Analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, though methods employing HPLC, liquid chromatography, immuno luminescence, and mass spectrometry are less common, but are becoming increasingly available (Hornung et al., 2015; DeVito et al., 1999; Baret and Fert, 1989; Spencer, 2013; Samanidou V.F et al., 2000; Rathmann D. et al., 2015). It is important to note that thyroid hormones concentrations can be influenced by a number of intrinsic and extrinsic factors (e.g., circadian rhythms, stress, food intake, housing, noise) (see for example, Döhler et al., 1979).

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

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

Short Name: T4 in neuronal tissue, Decreased

Key Event Component

Process	Object	Action
regulation of hormone levels	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent

AOP ID and Name	Event Type
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent

Stressors

Name
Methimazole
Propylthiouracil

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

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

Key Event Description

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

and cerebellum, T3 remains low throughout early and mid-gestation and corresponds with high activity of DIO3 in these brain regions. In late gestation and after birth, DIO3 levels drop in hippocampus and cerebellum with a corresponding increase in T3 concentrations (Kester et al., 2004).

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

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

Most of the hormone transported across the blood brain barrier is in the form of T4, primarily through the cellular membrane transporters (e.g., OATP1c1 transporter) into the astrocyte (Visser and Visser, 2012; Sugiyama et al., 2003; Tohyama et al., 2004). Within the astrocyte, T4 is converted into T3 via the local activity of deiodinase 2 (DIO2) (Guadano-Ferraz et al., 1997). A small amount of T3 may cross the blood brain barrier directly via the T3-specific transporter, MCT8 (Heuer et al., 2005). Although in mature brain T3 derives partially from the circulation and from the deiodination of T4, in the fetal brain T3 is exclusively a product of T4 deiodination (Calvo et al., 1990; Grijota-Martinez et al., 2011). In both cases, only the required amount of T3 is utilized in neurons and the excess is degraded by the neuron-specific deiodinase DIO3 (Tu et al., 1999; St. Germain et al., 2009; Hernandez et al., 2010).

Both deiodinase and transporter expression in brain peak in different brain regions at different times in fetal and neonatal life (Kester et al., 2004; Bates et al., 1999; Muller and Heuer, 2014; Heuer, 2007). Collectively, these spatial and temporal patterns of transporter expression and deiodinase activity provide exquisite control of brain T3 available for nuclear receptor activation and regulated gene expression.

How it is Measured or Detected

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

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Event: 756: Hippocampal gene expression, Altered

Short Name: Hippocampal gene expression, Altered

Key Event Component

Process	Object	Action
regulation of gene expression	hippocampal formation	abnormal

AOPs Including This Key Event

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

Stressors

Name
Methimazole
Propylthiouracil

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

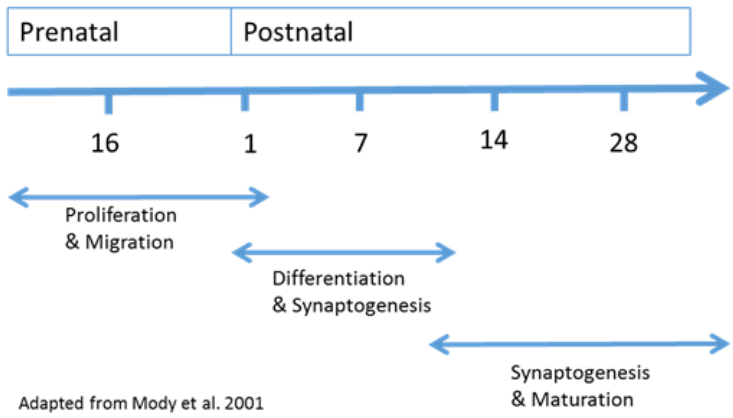
Sex	Evidence
Female	High
Male	High

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

Key Event Description

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

Figure 1. Mouse Hippocampal Developmental Stages Controlled by Gene Expression



During the very early prenatal period, genes corresponding to general cellular function are prominent (Mody et al., 2001). These are followed in time by genes regulating neuronal differentiation and migration in the mid to late gestational period. From late gestation (gestational day 15) until birth almost all the cells in the CA fields switch from a highly active proliferation state to a postmitotic state, and then undergo differentiation and migration. Expression of proliferative genes involved in cell cycle progression

are highly expressed at gestational day 16, then subsequently are silent immediately after birth when genes directing neuronal growth switch on. The pyramidal neurons of the CA fields in the hippocampus proper develop in advance of the granule cells that comprise the principal cells of the dentate gyrus. As such, the genes controlling the distinct phases of neurodevelopment are expressed at different times in these two hippocampal subregions (Altman and Bayer, 1990a; b). In both subregions, however, many phenotypic changes within the hippocampal neuron occur in the period immediately after birth (postnatal day 1 to 7). Almost all neurons show extensive growth and differentiation during the first postnatal week. These cellular changes are marked by rapid cytoskeletal changes, production of cell adhesion molecules, and extracellular matrix formation. The gene families involved in these processes include actins, tubulins, and chaperonin proteins essential for promoting correct protein folding of cytoskeletal components. Cell adhesion and extracellular matrix proteins are also upregulated during this period as these genes are critical for differentiation and synaptogenesis.

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

How it is Measured or Detected

Measurement of genomic profiles in developing brain use methods that are well established and accepted in the published literature. Microarray studies with expression profile analyses have been conducted in cortex and hippocampus of humans (Zhang et al., 2002), non-human primates, and rodent brains of various ages (Mody et al., 2001; Royland et al., 2008; Dong et al., 2015). More commonly, quantitative rtPCR or in situ hybridization have been used to probe individual gene transcripts (Dowling et al., 2000, Morte et al., 2010) or their protein products (Alvarez-Dolado et al., 1994; Gilbert et al., 2007). Recently RNA-Seq technology was applied to T3-treated primary mouse cortical cells and gene targets enriched in astrocytes and neurons to identify TH-responsive genes (Gil-Ibanez et al, 2015).

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Event: 757: Hippocampal anatomy, Altered

Short Name: Hippocampal anatomy, Altered

Key Event Component

Process	Object	Action
brain development	hippocampal formation	morphological change

AOPs Including This Key Event

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

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

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

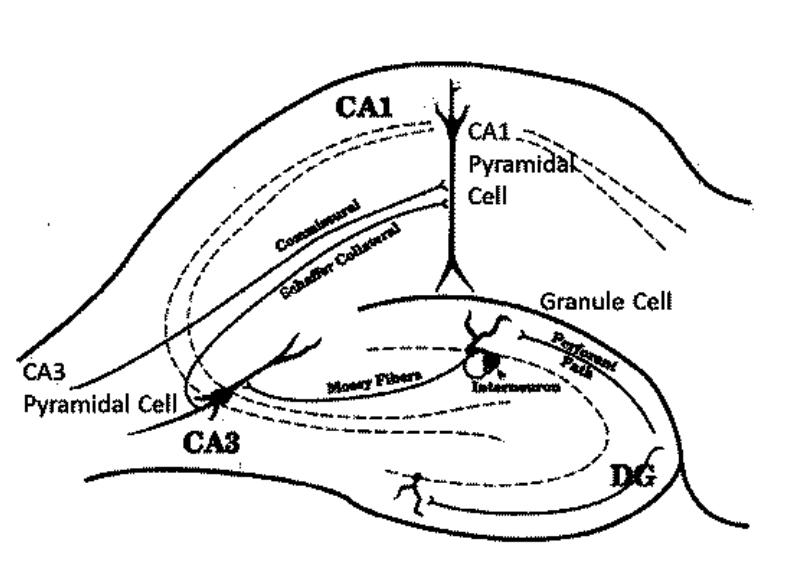
Key Event Description

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

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

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

Trisynaptic Hippocampal Circuitry



How it is Measured or Detected

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

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

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Event: 758: Hippocampal Physiology, Altered

Short Name: Hippocampal Physiology, Altered

Key Event Component

Process	Object	Action
chemical synaptic transmission	synapse	abnormal

AOPs Including This Key Event

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

Stressors

Name
Propylthiouracil
Iodine deficiency
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

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

Key Event Description

The hippocampus functions as a highly integrated and organized communication and information processing network with millions of interconnections among its constitutive neurons. Neurons in the hippocampus and throughout the brain transmit and receive

information largely through chemical transmission across the synaptic cleft, the space where the specialized ending of the presynaptic axon terminus of the transmitting neuron meets the specialized postsynaptic region of the neuron that is receiving that information (Kandell et al., 2012).

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

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

How it is Measured or Detected

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

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

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

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

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

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

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List of Adverse Outcomes in this AOP

Event: 402: Cognitive Function, Decreased

Short Name: Cognitive Function, Decreased

Key Event Component

Process	Object	Action
learning or memory		decreased
cognition		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	AdverseOutcome

AOP ID and Name	Event Type
Aop:300 - Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	AdverseOutcome

Stressors

Name
Methimazole
Propylthiouracil
Iodine deficiency

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

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

Key Event Description

Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition of, or retrieval of, a learned event, the hippocampal-based memory systems have received the most study. The main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990; Squire, 2004).

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

How it is Measured or Detected

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

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

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

3) Contextual Fear conditioning is a hippocampal based learning task in which animals are placed in a novel environment and allowed to explore for several minutes before delivery of an aversive stimulus, typically a mild foot shock. Upon reintroduction to this same environment in the future (typically 24-48 hours after original training), animals will limit their exploration, the context of this chamber being associated with an aversive event. The degree of suppression of activity after training is taken as evidence of retention, i.e., memory (Curzon et al., 2009).

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

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

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

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

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

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

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

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

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

Regulatory Significance of the AO

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

1998; OECD, 2007).

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Appendix 2

List of Key Event Relationships in the AOP

There are no Relationships associated with this AOP