Developmental Toxicity of Perfluorohexane Sulfonate (PFHxS) - Effects on the Immune and Thyroid Hormone Systems

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Developmental Toxicity of Perfluorohexane Sulfonate (PFHxS)

- Effects on the Immune and Thyroid Hormone Systems

PhD Thesis

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PhD Thesis

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Preface

This thesis is submitted as part of the requirements for obtaining the PhD degree at the Technical University of Denmark. The presented work has been carried out at the National Food Institute, Technical University of Denmark and in the Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, North Carolina. My work has been supervised by Senior Researcher Marta Axelstad, Professor Ulla Hass and Senior Scientist Charlotte Bernhard Madsen.

The experimental work was funded by larger projects from the Danish Centre on Endocrine Disrupters (CeHoS), and the Danish Environmental Protection Agency, Ministry of Environment and Food of Denmark.

Louise Ramhøj
Kgs. Lyngby, June 2018

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I would like to thank Terje Svingen and Maj Sahlholt for corrections for this thesis. Last, I would like to thank my friends and family for putting up with me during this journey.

Summary

Both the immune and the thyroid hormone systems are integral to human physiology and health. They are also important for normal development from early in fetal life, but simultaneously vulnerable to chemical exposures and disruption during fetal and early postnatal life. If disrupted during development, both systems can become compromised leading to negative consequences later in life. This includes effects on immune function, neurobehavior and cognitive abilities. In fact, since thyroid hormones are crucial for brain development there are correlations between low maternal serum thyroxine (T4) levels and subsequent adverse effects on child neurobehavior and intelligence quotient (IQ).

Poly- and perfluoroalkylic substances (PFAS) are compounds linked to immunotoxicity and thyroid hormone disruption. They are ubiquitous and persistent in the environment and human exposure is universal, hence a potential risk to human health. Yet the toxicity potential of several PFASs, including perfluorohexane sulfonate (PFHxS), remains largely unknown. This PhD project therefore aimed at investigating the developmental toxicity of PFHxS, with an emphasis on the reproductive, the thyroid hormone and the immune systems. PFHxS toxicity was further investigated in combination with exposure to a human-relevant mixture of 12 endocrine disrupting chemicals. These studies showed that, for some endpoints, there was increased sensitivity to PFHxS exposure when it was combined with the mixture and that PFHxS and the mixture could potentiate the effect of each other. This indicates that the current European approach for regulating chemicals,

relying on risk assessment of one chemical at a time, may underestimate the risk to humans who are exposed to a multitude of chemicals on a daily basis.

The main finding in these studies was that PFHxS reduced maternal and offspring thyroid hormone levels. This happened without apparent activation of the compensatory feedback responses. We were not able to correlate the decreased thyroid hormone levels to behavioral effects in the offspring, but the literature suggests that the employed tests are insensitive measures of altered brain development due to developmental thyroid hormone reductions. In humans, however, even smaller reductions in thyroid hormone levels during development, may affect complex functional parameters as language development and IQ, endpoints which are not measurable in rodents. Hence, in order to protect human brain development there is a strong need for development of new test methods for identification of adverse effects on brain development due to disruption of the thyroid hormone system. Preferably, these would include assays that are sensitive to more moderate reductions of thyroid hormone levels (reduction in T4<50%). Such tests are necessary because the legislative framework for regulating the use of chemicals relies on assays showing downstream adverse outcomes. Until a suitable test for adverse effects due to developmental thyroid hormone disruption has been identified, a compromise designed to protect human cognition could be to regard decreased serum T4 as an adverse outcome in itself.

PFHxS exposure had limited effects on the immune system in our study; however, a faulty study design impedes conclusions on especially functional immune endpoints, which in theory should be the most sensitive measure of immune competence. At the highest tested dose, PFHxS may reduce immune organ weights, but this could not be replicated in the larger study that investigated lower doses. Hence, further studies of immunotoxicity of PFHxS are warranted.

Resumé

Både immun- og thyreoideahormon-systemerne er vigtige for human fysiologi og sundhed. Fra tidligt i fosterstadiet er de også vigtige for en normal udvikling, men samtidigt yderst sårbare over for påvirkning fra kemikalier. Hvis der sker forstyrrelser under udviklingen kan begge systemer blive påvirkede, og det kan blandt andet have negative konsekvenser for immunfunktionen, adfærd og kognitive evner senere i livet. De rette niveauer af thyreoideahormonerne er afgørende for hjernens udvikling, og mange studier har vist sammenhænge mellem lave thyroxin(T4)-niveauer i gravide og skadelige effekter på deres børns adfærd og intelligenskvotient (IQ).

Poly-og perfluorerede stoffer (PFAS) er blevet kædet sammen med immuntoksicitet og thyreoideahormonforstyrrelser. Stofferne udgør en potentiel sundhedsrisiko for mennesker, da de findes overalt i miljøet, er persistente, og menneskers udsættelse for stofferne er universel. På trods af dette, er der stadig adskillige PFASer, hvis toksicitet er stort set ukendt. Det gælder også perfluorhexansulfonat (PFHxS). Dette PhD-projekt har derfor haft til formål, ved hjælp af rotteforsøg, at undersøge PFHxSs toksiske effekter på udviklingen, med et særligt fokus på effekter i reproduktions-, thyreoideahormon- og immunsystemet. Derudover blev PFHxS undersøgt for kombinationseffekter ved at udsætte rotterne for både PFHxS og en blanding af 12 hormonforstyrrende kemikalier, som er repræsentative for, hvad mennesker udsættes for i dagligdagen. Studierne viste, at udsættelse for PFHxS i kombination med blandingen af miljøkemikalier førte til øget toksicitet af PFHxS for nogle effekter, og at PFHxS og blandingen af miljøkemikalier kunne potentiere effekten af

hinanden. Dette indikerer, at den nuværende europæiske metode til regulering af kemikalier, hvor risikovurdering foretages for et kemikalie ad gangen, kan undervurdere risikoen for mennesker, som jo i dagligdagen udsættes for en kompleks blanding af kemikalier.

Det vigtigste fund i de udførte dyrestudier var at PFHxS nedsatte niveauet af thyreoideahormoner hos både mødre og unger. Der sås ikke nogen tydelig aktivering af hormonaksens feed-back mekanismer. Der var ikke nogen klar sammenhæng mellem de nedsatte thyreoideahormonniveauer under udviklingen og adfærdseffekter i ungerne, men ud fra litteraturen tyder det på, at de anvendte tests er ufølsomme over for ændret hjerneudvikling der er opstået som konsekvens af thyreoideahormonforstyrrelser under udviklingen. Hos gravide kvinder ses mindre nedsættelser i T4 niveauerne imidlertid at have en effekt på komplekse funktionelle parametre som sprogudvikling og IQ hos deres børn, hvilket jo ikke kan måles i rotter. For at kunne beskytte hjerneudviklingen hos mennesker er der derfor et stort behov for udvikling af nye testmetoder, så man i dyreforsøg kan måle effekter på hjerneudviklingen som følge af påvirkning af thyreoidea-hormonsystemet. Der er især et behov for tests som er følsomme overfor moderate nedsættelser i thyreoideahormonniveauet (T4 reduktion<50%). Sådanne tests er helt nødvendige, fordi brugen af hormonforstyrrende kemikalier reguleres på baggrund af anerkendte tests, der påviseligt har ført til skadelige effekter i dyr. Indtil sådanne tests for skadelige effekter i forsøgsdyr er udviklet, kan et kompromis for at beskytte hjerneudvikling hos mennesker være, at anse nedsættelse af T4-niveauet som en skadelig effekt i sig selv.

I vores studie viste udsættelse for PFHxS begrænsede effekter på immunsystemet. Men et ikke optimalt studiedesign forhindrede desværre, at der kunne drages konklusioner på især de funktionelle parametre, som i teorien skulle være de mest følsomme indikatorer for immunfunktionen. Ved den højest undersøgte dosis tydede det på, at udsættelse for PFHxS nedsatte vægten af immunorganer, men dette kunne ikke bekræftes i det store forsøg, som blev udført med lavere doser. Derfor vil yderligere studier af PFHxS's mulige immuntoksicitet være nødvendige.

List of manuscripts

Papers and manuscripts included in thesis

Manuscript I **Ramhøj L**, Hass U, Boberg J, Scholze M, Christiansen S, Nielsen F, Axelstad M, (2018). Perfluorohexane Sulfonate (PFHxS) and a Mixture of Endocrine Disrupters Reduce Thyroxine Levels and Cause Antiandrogenic Effects in Rats, *Toxicological Sciences*, 163(2), p. 579–591.

Manuscript II **Ramhøj L**, Hass U, Gilbert ME, Wood C, Usai D, Vinggaard AM, Mandrup K, Christiansen S, Svingen T, Axelstad M, Evaluation of Thyroid and Neurodevelopmental Effects in Rats after Perinatal Exposure to Perfluorohexane Sulfonate (PFHxS), *manuscript.*

Manuscript III **Ramhøj L**, Hass U, Madsen CB, Developmental Immunotoxicity of Perfluorohexane Sulfonate (PFHxS) – a Study Report. *Study report.*

Additional Publications

Svingen T, **Ramhøj L**, Egebjerg KM, Christiansen S, Petersen MA, Vinggaard AM, Hass U, (2018), Effects on metabolic parameters in young rats born with low birth weight after exposure to a mixture of pesticides, Scientific Reports. 8, 305.

Kronborg TM, Hansen JF, Rasmussen ÅK, Vorkamp K, Nielsen CH, Frederiksen M, Hofman-Bang J, Hahn CH, **Ramhøj L**, Feldt-Rasmussen U, (2017), The flame retardant DE-71 (a mixture of polybrominated diphenyl ethers) inhibits human differentiated thyroid cell function in vitro. PLoS ONE, 12(6).

Kronborg, TM, Hansen, JF, Nielsen CH, **Ramhøj L**, Frederiksen M, Vorkamp K, Feldt-Rasmussen U, (2016), Effects of the Commercial Flame Retardant Mixture DE-71 on Cytokine Production by Human Immune Cells, PLoS ONE, 11(4).

Abbreviations

3-MC	3-methylcholanthrene
ab	Antibody
ADHD	Attention Deficit Hyperactivity Disorder
AGD	Anogenital distance
ANOVA	Analyzed by analysis of variance
AOP	Adverse Outcome Pathway
BMI	Body mass Index
CAR	Constitutive androstane receptor
CeHoS	Danish Centre on Endocrine Disrupters
CoV	Coefficient of variations
D1	Deiodinases type 1
D2	Deiodinases type 2
D3	Deiodinases type 3
DTH	Delayed type hypersensitivity

ECLIA	Electrochemiluminescence-immunoassay
EDC	Endocrine disrupting chemical
EDmix	A mixture of 12 endocrine disrupting chemicals, described in chapter 3
ELISA	Enzyme-linked immuno sorbent Assay
EOGRTS	Extended One Generation Reproductive Toxicity Study
EPA	Environmental Protection Agency
fT4	free T4
GD	Gestation day
hCG	Human chorionic gonadotropin
HPT	Hypothalamic-pituitary-thyroid axis
I	Iodide
IDD	Iodine deficiency disorder
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQ	Intelligence quotient
KE	Key event
KER	Key event relationship
KLH	Keyhole limpet hemocyanin
MCT	Monocarboxylate transporter
MIE	Molecular initiating event
MMI	Methimazole
MMR	Measles mumps and rubella
MoA	Mode of Action
MRI	Magnetic resonance imaging

mRNA	messenger ribonucleic acid
NIS	Sodium-Iodide Symporter
NF-κB	Nuclear factor-κB
NK	Natural killer
NOAEL	No observed adverse effect level
NR	Nipple retention
NTP	National Toxicology Program
OATP	Organic anion transporting polypeptide
OECD	Organisation for Economic Co-operation and Development
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCN	Pregnenolone-16α-carbonitrile
PD	Pup day
PFAS	Poly- and perfluoroalkylic substances
PFBS	Perfluorobutane sulfonate
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	
PFOA	Perfluorooctanoic acid
PFOS	Perflurooctane sulfonate
PND	Postnatal day
POP	Persistent Organic Pollutant
PPAR	Peroxisome proliferator-activated receptor
PSU	Polysulfone

PTU	Propulthiouracil
Px	Perfluorohexane sulfonate (PFHxS)
PXR	Pregnane X receptor
SDU	University of Southern Denmark
SULT	Sulfotransferase
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyrosin-binding globulin
TDAR	T-cell dependent antigen
Tg	Thyroglobulin
TH	Thyroid hormones
Th2	T helper cell type 2
TPO	Thyroperoxidase
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
UGT	UDP-Glucuronosyltransferases
UDP-Glucuronosyltransferases	Uridine 5'-diphospho-glucuronosyltransferase
vPvB	Very persistent and very bioaccumulative
WHO	World Health Organisation

Table of contents

1 Introduction

This chapter introduces the topic of the thesis (1.1), the rationale and aims (1.2) and provides an outline (1.3) of the following chapters.

1.1 Introduction

The last decades have witnessed a rising prevalence of disorders of the immune and thyroid systems such as asthma, allergies, autoimmune disorders and thyroid cancers [A. C. Gore et al., 2015; Lim et al., 2017; McFadden et al., 2015; WHO/UNEP, 2013]. Neurobehavioral disorders are also increasing, and in the U.S. an estimated 1 in 59 children are currently being diagnosed with autism spectrum disorders and 9.4% with attentive hyperactivity disorders [Baio et al., 2018; Danielson et al., 2018]. The rate at which this surge in incidents have occurred excludes genetic factors as the primary cause, but rather suggests a combination of changing diagnostic patterns and environmental factors to be the cause. Early life exposure to environmental chemicals could constitute such an environmental factor as both the immune and thyroid hormone systems seem particularly vulnerable to xenobiotic disruptions during development [Dietert, 2008; Gilbert & Zoeller, 2011].

Both the thyroid hormone and immune systems are essential for maintaining organismal homeostasis and health in adult life, but to function optimally they rely on a complex network of tissues, organs and inter-related signaling pathways. The foundation for this network is set down during fetal and early postnatal life as the simple zygote grows and develops into a fully formed organism. Exposure to xenobiotics during these sensitive life-stages can disrupt the process and cause life-long consequences [A. C. Gore et al., 2015; WHO/UNEP, 2013]. With regard to the immune system, disrupted development could render the offspring predisposed to immune dysfunction and non-communicable diseases. As for the thyroid hormone system, a primary concern is that the brain does not develop optimally, since thyroid hormones are crucial for development of the central nervous system.

Perfluorinated compounds are one class of chemicals that have been implicated in immunotoxicity and thyroid hormone disruption. Much of the available data comes from human epidemiological studies however, and empirical cause-effect relationship data remain largely lacking. This knowledge gap was a major motivation for this PhD project, which sought to better explain how, and if developmental exposure to perfluorinated chemicals can have detrimental consequences for the offspring's immune- or neurocognitive status.

1.2 Rationale and aims

The aim of this project was to investigate the developmental toxicity of perfluorohexane sulfonate (PFHxS), using the rat as a model. Focus was on the immune and thyroid hormone systems, guided by epidemiological studies. For instance, correlations between PFHxS and human immune function has implicated the immune system as a potential target of PFHxS, whereas studies on thyroid function and the importance for brain development has shown the need for increased understanding of thyroid hormone disruptors and their adverse effects on brain development in animal studies.

1.3 Outline

With chapter 1 providing the introduction to this thesis, chapter 2 contains the background for the scientific work. It introduces the perfluorinated compounds, reviews the literature that prompted us to perform the studies on PFHxS. Additionally it contains an introduction to the thyroid hormone system and thyroid hormone disrupting compounds, and reviews the literature on human thyroid hormone status and implications for brain development. Chapter 3 contains the scientific paper on developmental toxicity of PFHxS where the most important finding was effects on thyroid hormone levels in serum. In chapter 4, the manuscript focuses on this identified effect and studies the thyroid hormone system and potential adverse effects in response to thyroid hormone disruption by PFHxS. Chapter 5 contains the study report on the immunotoxicological work. In chapter 6 the results of the presented work are discussed and contrasted to previous work on thyroid hormone disrupting chemicals and the potential implications for human brain development. Finally, chapter 7 contains the conclusions of this project.

2 Background

This chapter introduces the concept of environmental chemicals and endocrine disruptors (2.1), the perfluorinated compounds (2.2), the thyroid hormone system and thyroid hormone disruptors (2.3) and the importance of thyroid hormones during brain development (2.4). It concludes with a description of the influence of perfluorinated compounds on thyroid status (2.5) and the developing immune system (2.6).

2.1 Environmental chemicals and endocrine disruptors

Thousands of industrial chemicals are produced and a large number are now ubiquitous in the environment. Consequently, the combined human exposure to chemicals via foods, textiles, dust, and so forth, make up a complex mixture that potentially can influence development and health [Kortenkamp, 2014; Svingen & Vinggaard, 2016; WHO/UNEP, 2013].
Human exposure to environmental chemicals may cause a plethora of effects, including disruption of endocrine functions and adverse effects on the developing immune system. The World Health Organisation (WHO) has defined an endocrine disrupting chemical (EDC) to be "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations" [WHO/IPCS, 2002]. The identification of a chemical as an endocrine disruptor hence relies on both an identified endocrine mode of action causing a physiological change, and a subsequent adverse effect.

The majority of industrial chemicals have not been thoroughly tested for their potential endocrine disrupting potential prior to market introduction, and testing them all is extremely resource demanding [Svingen & Vinggaard, 2016]. In order to increase knowledge on toxicological pathways and make better use of knowledge generated via alternative approaches (e.g. non-animal tests), the Organisation for Economic Co-operation and Development (OECD) initiated a toxicological knowledge framework programme; Adverse Outcome Pathways (AOPs) to "support chemical risk assessment based on mechanistic reasoning" [OECD, n.d.]. An AOP describes causally linked events leading to an adverse health or ecotoxicological effect.

The work on thyroid hormone disruption performed in this thesis builds on the AOP framework and parallels some of the work performed at the US Environmental Protection Agency (EPA) for the AOP number 42 "Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals" (Fig. 1) [Crofton et al., 2018; Hassan et al., 2017]. This AOP initiates with inhibition of thyroid hormone synthesis (through thyroperoxidase inhibition) resulting in decreased serum thyroxine (T4) levels leading to reduced thyroid hormone mediated gene transcription in the brain, and subsequent effects on brain development and cognition. Decreased serum T4 was our point of entry into the AOP, it is a central Key Event shared by many thyroid hormone disrupting chemicals acting through different targets.

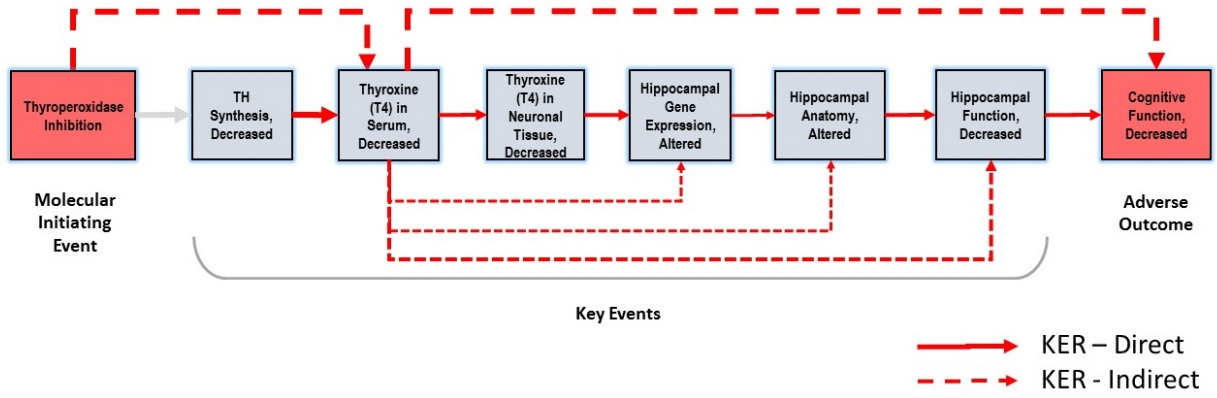

Figure 1 | Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals. The AOP describes the sequential relationship of events following inhibition of thyroperoxidase by a xenobiotic. The molecular initiating event leads to reduced serum thyroxine, reduced thyroxine in the developing brain, reduced thyroid hormone mediated gene expression and altered morphology and function resulting in the adverse outcome defined as decreased cognitive function. KER: Key event relationship, TH: Thyroid hormones. From [Crofton et al., 2018].

2.2 Perfluorinated compounds

Poly- and perfluoroalkylic substances (PFAS) comprise a large group of industrial chemicals used as surfactants since the 1950s, largely for their ability to repel oil and water. There are hundreds of PFAS that have been, or still are used in a variety of consumer products, from food contact materials and Teflon-coated frying pans, to upholstery and outdoor adventure gear. The industrial uses include firefighting foams, lubricants, paints and emulsifiers for fluoropolymer production [Lindstrom et al., 2011].

Although PFAS are desired for their oil and water repellency, it is exactly these properties that make them problematic environmental pollutants; they are persistent and bioaccumulate in animals and humans. The perfluorinated compounds are fully fluorinated on the carbon chain and the strength of these carbon-fluorine bonds prevent metabolism in humans. Instead it appears that they, unlike many other Persistent Organic Pollutants (POPs), are not stored in fatty tissues but instead bind to proteins and are distributed in blood, liver and peripheral tissues [Kerstner-Wood et al., 2003; Pérez et al., 2013].
The longstanding use and persistence of the compounds have ensured widespread environmental contamination. They are found at the bottom of the ocean, at the top of the atmosphere and in polar bears of remote arctic regions, as well as in virtually all tested individuals in tissues including brain, blood, milk, lungs, and liver [Calafat et al., 2007; Giesy & Kannan, 2001; Greaves et al., 2012; Gützkow et al., 2012; Kärrman et al., 2007; Mørck et al., 2015; Nøst et al., 2014; Smithwick et al., 2005].

Considerable efforts have been made to restrict the use of PFAS, and the use of especially the long-chained compounds now appear to have been reduced due to measures taken by a number of governments, users and the industry producing the compounds [Blum et al., 2015; Lindstrom et al., 2011; Stockholm Convention, 2009; US EPA, 2006, 2015]. This reduction in use has been confirmed by some epidemiological studies [Axmon et al., 2014; Bjerregaard-Olesen et al., 2016; Eriksson et al., 2017; Nøst et al., 2014; Schröter-Kermani et al., 2013; Seo et al., 2018], whereas it is not (yet) evident in a few others [Glynn et al., 2012; Kato et al., 2011; E. Okada et al., 2013]. Some uncertainties with regard to changing production patterns also exist and it has been suggested that production has moved to Asia and shifted to some of the more unknown compounds [Axmon et al., 2014; Eriksson et al., 2017; Nøst et al., 2014; Seo et al., 2018]. Nevertheless, contamination of the general environment and point sources and exposure of the general population will continue for years to come [Wang et al., 2017].

2.2.1 PFOS, PFOA and PFHxS

The human exposure to PFAS is widely documented. Perflurooctane sulfonate (PFOS) is typically found at the highest concentrations, often followed by perfluorooctanoic acid (PFOA) and PFHxS. PFOS and PFOA are by far the most studied perfluorinated compounds and also those that generally appear to be the most toxic (Fig. 2). Toxicities *in vitro*, in animals and in humans include effects on the metabolic-, immune-, and thyroid hormone systems. PFHxS with its C6-chain is at the boundary between what is classified

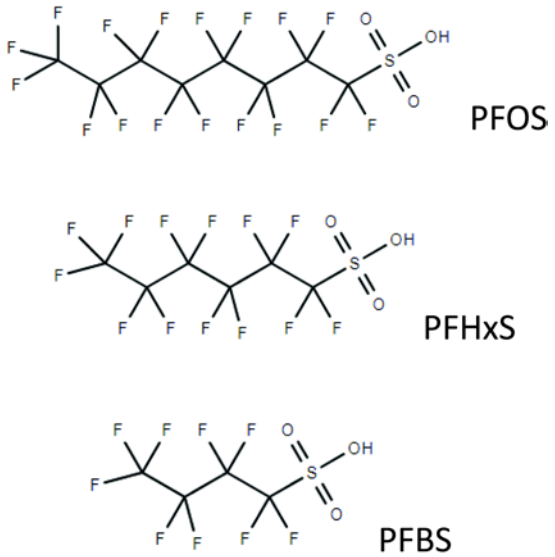

Figure 2 | Chemical structures of Perflurooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS) and perfluorobutane sulfonate (PFBS).

as short or long-chain compounds, since its acid counterpart perfluorohexanoic acid (PFHxA) is a short chain PFAS, while PFHxS is usually termed a long-chained PFAS [Buck et al., 2011]. PFHxS is interesting with regard to human toxicity for a number of reasons. It is usually in the top 3 of human exposures and its half-life stands out in, likely comparison to the other PFAS where it does not follow the norm with decreasing chain length predicting decreased half-life. Rather, the order is PFHxS>PFOS>perfluorobutane sulfonate (PFBS). The half-life of PFHxS is longer than 7 years in humans [Axmon et al., 2014; Gao et al., 2015; Olsen et al., 2007; Yang et al., 2016] due to enterohepatic circulation of PFAS [Genuis et al., 2013; Zhao et al., 2015, 2017]. There are also reports suggesting that some sites are contaminated with the compound [Gyllenhammar et al., 2015; Hu et al., 2016; Norström et al., 2015] and PFHxS has recently been classified according to the European chemicals legislation, REACH, as a substance of very high concern, due to its very persistent and very bioaccumulative (vPvB) properties [ECHA, 2017a, 2017b, 2017d]. Furthermore, it is proposed for listing as a persistent organic pollutant (POP) according to the Stockholm Convention [UNEP, n.d.]. In spite of this, the toxicity of PFHxS is not well described [Danish Ministry of the Environment, 2015; FSANZ, 2016] and at the initiation of this project only one developmental toxicity study in rats could be identified [Butenhoff, Chang, et al., 2009], warranting further studies into how PFHxS might affect exposed individuals in the future. A study in mice has recently been published [S. Chang et al., 2018].

2.3 The thyroid hormone system and thyroid hormone disruptors

The main function of the two thyroid hormones is to regulate metabolism. They exert their actions in most tissues and organs of the mammalian organism. During development their function is more dynamic and they have a crucial role in the development of the central nervous system where they help regulate neurogenesis, migration, synaptogenesis and myelination [Gilbert & Zoeller, 2011; Howdeshell, 2002; Moog et al., 2017]. Because of the complexity of the thyroid hormone system, chemicals can interfere with thyroid hormone action in many different ways and in various target tissues.

2.3.1 The thyroid hormone system

Thyroid hormone action is controlled by an intricate system making up the thyroid hormone system. Central is the hypothalamic-pituitary-thyroid axis (HPT-axis) (fig. 3). Here thyrotropin-releasing hormone (TRH) from the hypothalamus stimulates the release of thyroid-stimulating hormone (TSH) from the pituitary. TSH stimulates the thyroid follicular cells through the TSH-receptor to increase activity of the thyroid gland. This includes all steps of TH synthesis and secretion and both increased expression and activity of enzymes. Iodide uptake is increased through the Sodium-Iodide Symporter (NIS), and hormone production and excretion is stimulated. Thyroperoxidase (TPO) performs the rate-limiting step of hormone synthesis that occurs in the thyroid follicle lumen [Ortiga-Carvalho et al., 2016]. TPO incorporates iodine into thyroglobulin (Tg) forming iodothyrosine scaffolds, on which the thyroid hormones (TH) thyroxine (T4) and triiodothyronine (T3) are built and released to the circulation in a ratio of 80:20 [Laurberg, 1976]. T4 and T3 in turn exert negative feedback on release of TRH and TSH from the hypothalamus and pituitary, to control thyroid hormone homeostasis [Ortiga-Carvalho et al., 2016].
THs are released to the circulation from the thyroid gland, where they bind to the serum distributor proteins albumin, transthyretin (TTR) and thyrosin-binding globulin (TBG). These proteins are believed to transport and shield thyroid hormones from metabolism and transport them to their target cells [Alshehri et al., 2015; Mendel et al., 1987]. The fraction (<1%) of unbound free hormone is in equilibrium with the much larger pool of protein bound hormones [Alshehri et al., 2015]. Assays measuring free T4 (fT4) intends to

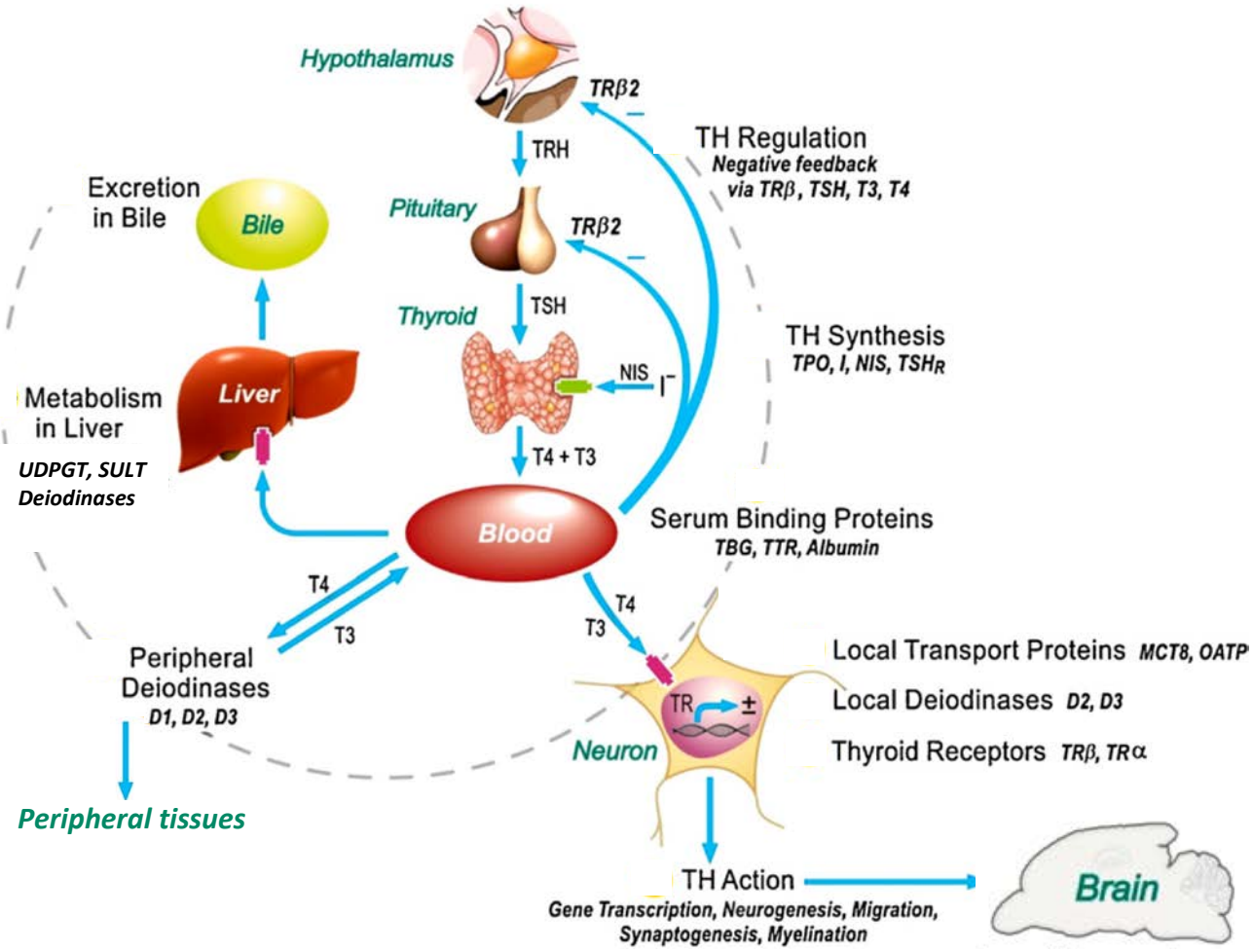

Figure 3 | Overview of the hypothalamic-pituitary-thyroid axis and the thyroid hormone system with points of regulation. See text for details. TR: Thyroid Receptor, TRH: Thyrotropin-Releasing Hormone, TSH: Thyroid-Stimulating Hormone (thyrotropin), NIS: Sodium-Iodide Symporter (mediates uptake of Iodide into the thyroid follicular cells), I^-: Iodide, T4: Thyroxine, T3: Triiodothyronine, TH: Thyroid Hormone, TPO: Thyroid Peroxidase (oxidation of Iodide to iodine, TSH_R: TSH receptor, TBG: Thyroxine-binding globulin, TTR: Transthyretin, MCT8: Monocarboxylate Transporter (specific T3 transporter), OATP: Organic Anion Transporting Polypeptide, D1: Deiodinase type I, D2: Deiodinase type II, D3: deiodinase type III, UDPGT: Uridinediphosphate-Glucoronosyl Transferases, SULT: Sulfotransferases. Modified from [Gilbert et al., 2012].

determine the amount of fT4 while measurements of total T4 include both the free and protein bound T4.

Thyroid hormones are actively transported into cells through membrane transporters as monocarboxylate transporter (MCT) 8 and organic anion transporters (OATPs) [Bernal et al., 2015; Landers & Richard, 2017; Wirth et al., 2014]. Here, deiodinases convert T4 to the active T3 which acts on the nuclear thyroid hormone receptors (TR)s in target cells, these are ligand-regulated transcription factors working with accessory proteins to repress or promote gene transcription [Schroeder & Privalsky, 2014]. Accordingly, lack of receptor ligand has a function and as such can have a more detrimental effect on development than simple lack of the entire receptor [Gil-Ibañez et al., 2013; Sjögren et al., 2007]. Differential expression of the receptor isoforms secures some of the regulation of TH tissue action during development [Bradley et al., 1992].

The complexity of the thyroid system ensures tight control and extensive possibilities of spatiotemporal action of the hormones. This can be observed in the developing brain where action is tightly controlled for both time and place on a single cell level [Zoeller, 2010]. However, this also provides ample opportunity for environmental chemicals and drugs to interfere with thyroid hormone action. Chemicals and drugs have been shown to act on various targets as TPO, NIS, serum distributor proteins, transporters, liver enzymes, deiodinases, and TRs [Crofton, 2008; A. C. Gore et al., 2015; Howdeshell, 2002; WHO/UNEP, 2013; Zoeller, 2007].

2.3.2 Metabolism of thyroid hormones

Deiodinases

The deiodinases are involved in TH metabolism by activating and deactivating TH (fig.4). Most peripheral tissues are dependent upon peripheral activation of T4 to T3 by deiodinase type 1 (D1), the brain being an exception (see below). As deiodinases also degrades TH, the expression and activity of the enzymes are used spatiotemporally to ensure TH action locally, irrespective of the circulating hormone levels, and to secure against hormone excess [Bates et al., 1999; Hernandez et al., 2010, 2012; Zoeller, 2010].
The deiodinases forms part of the compensatory mechanisms of the thyroid system. D1 expression in liver is negatively regulated by TH, so that expression is increased in

hypothyroidism [Zavacki et al., 2005]. Whereas D1 expression in thyroid is proportional to serum T4 levels and hence is impaired in hypothyroidism. Hypothyroidism also increases brain D2 to maintain T3 levels as seen by PCB induced hypothyroxinemia in fetal rats and iodine deficiency [Morse et al., 1993; Peeters et al., 2001].

Liver enzymes

Degradation and excretion of thyroid hormones are taken care of by uridine 5'-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferases, UGTs), sulfotransferases (SULTs) and deiodinases (D1, D2 and D3) working in concert (fig. 4). While sulfation primarily marks T3 and T4 for inactivation by D1 [Visser, 1994], glucuronidation facilitate direct biliary excretion of the hormones. It is believed that the UGT1A family of enzymes perform glucuronidation of T4 while the UGT2Bs have T3 as substrate [T. A. Richardson & Klaassen, 2010a, 2010b].

Some thyroid hormone disrupting compounds act as microsomal enzyme inducers which can increase the activity of UGTs and SULTs resulting in increased excretion of TH with a subsequent fall in serum levels of primarily T4 [Hood & Klaassen, 2000a; Klaassen & Hood, 2001; McClain, 1989; Visser et al., 1993].

With some microsomal enzyme inducers the HPT axis is activated and TSH is increased leading to effect on the thyroid gland: follicular cell differentiation, hypertrophy, reduced colloid, and increased gland weight [Hood et al., 1999; Klaassen & Hood, 2001] as would be expected based on the theory of the negative feedback loop. However, the reason for this increase in TSH remains elusive as not all microsomal enzyme inducers actually cause this expected effect on TSH. Instead, comparative studies of enzymes inducers have shown that the increased TSH is not correlated with decreased circulating levels of T4 or T3, and not to T4 glucuronidation. Potentially, it is mediated through increased biliary excretion following glucuronidation of T3 by unknown enzymes that are induced by pregnenolone-16α-carbonitrile (PCN) but not by 3-methylcholanthrene (3-MC) or Arochlor 1254 (a polychlorinated biphenyl, PCB). These compounds all reduce T4, but cause no effect on TSH [Hood & Klaassen, 2000a; T. A. Richardson & Klaassen, 2010b; Vansell & Klaassen, 2001, 2002a, 2002b]. These studies also showed marked differences in the effect patterns by microsomal enzyme inducers: serum total and free T4 and T3, TSH, T4 glucuronidation and half-life, T3 glucuronidation, thyroid gland effects, D1 activity in liver, kidney and thyroid, and D2 activity in brown adipose tissue, pituitary and brain, can all be differentially affected by different compounds [de Sandro et al., 1991; Hood et al., 1999; Hood & Klaassen, 2000a, 2000b; Klaassen & Hood, 2001; T. A. Richardson & Klaassen, 2010a, 2010b; Visser et al., 1993]. Additionally, there is a wide range of compensational effects

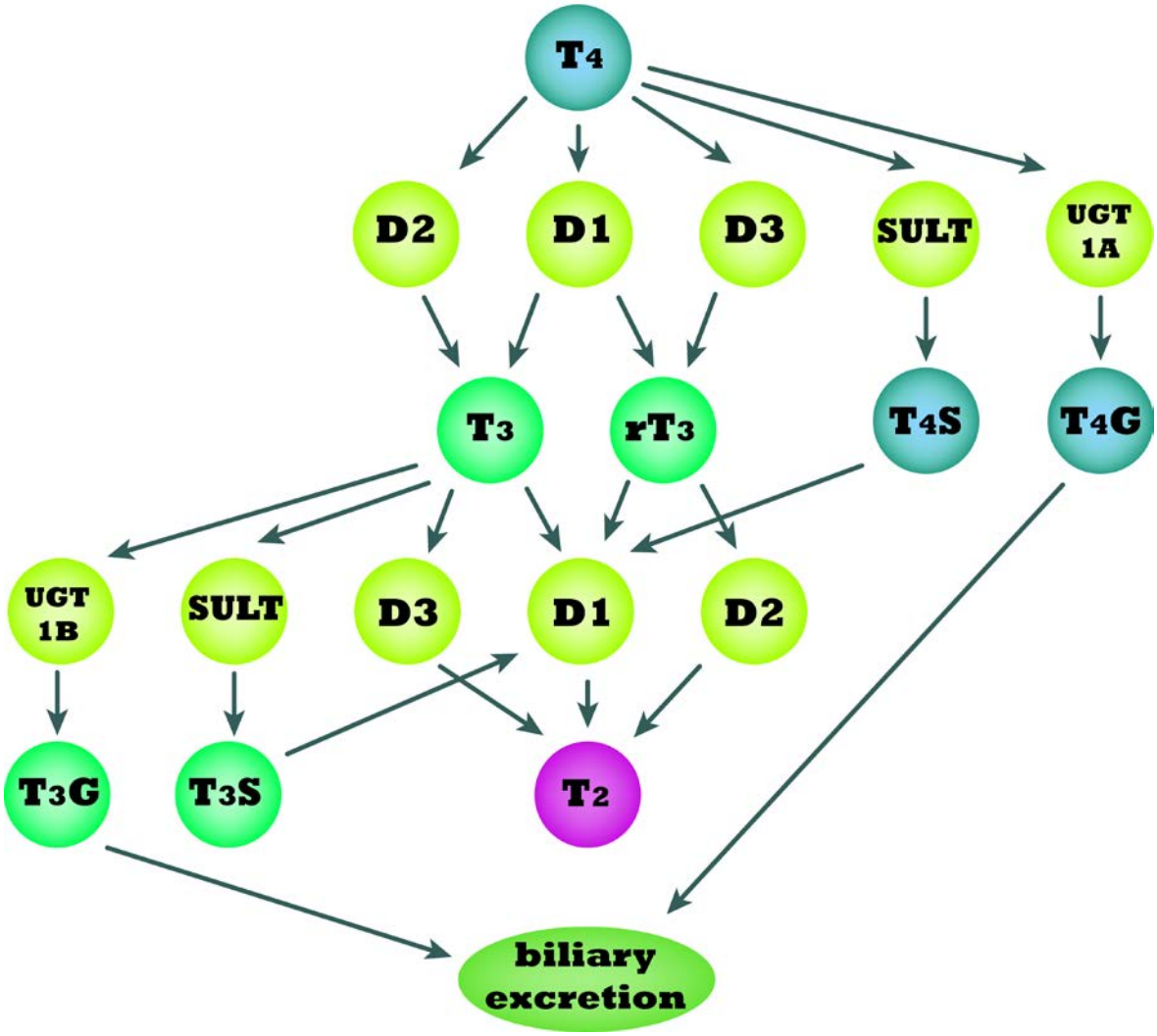

Figure 4 | Major pathways for thyroid hormone metabolism. See text for details. T4: thyroxine, T3: tri-iodothyronine, rT3: reverse T3, T2: diiodothyronine, D1,D2 and D3: deiodinase type 1,2 and 3, SULT: sulfotransferases, UGT: uridine diphosphate glucuronosyltransferases, T4G: T4-glucuronide, T4S: T4-sulfate, T3G: T3-glucuronide, T3S: T3 sulfate.

and additional pathways that may be activated as shown by studies of Ugta1 deficient Gunn rats and Ugt2b2 deficient Fischer rats [T. A. Richardson & Klaassen, 2010a, 2010b]. This clearly shows that there is not one prototypic microsomal enzyme inducer, but rather indicates that the different compounds either directly cause the observed differences or that the organism responds differentially to different chemical insults. Furthermore, additional compensatory responses in other parts of the thyroid system may also be affected. Effects

on hepatic transporters, MCT8, D3, thyroid gland genes, distributor protein levels and TRH and TSH receptors in hypothalamus and thyroid [Liu et al., 2015; V. M. Richardson et al., 2008; Wu et al., 2016] may all contribute to the observed phenotype.

Compounds which can displace TH from the serum distributor proteins are also believed to cause increased thyroid hormone metabolism. Binding of compounds to distributor proteins can displace thyroid hormone form the proteins, increasing the free fraction of the hormones and making them available to metabolizing enzymes [Alshehri et al., 2015; Chanoine et al., 1992]. It is not known whether this displacement of TH in turn can cause liver enzyme induction, potentially further decreasing circulating hormone levels; many distributor protein binders appear to both cause enzyme induction and displace T4 but it is uncertain whether this is due to multiple modes of action or just the protein binding with derived additional effects.

2.3.3 Transport of thyroid hormones to the fetal brain

Thyroid hormones are functional from early in gestation. They are potentially available to the rat fetus from GD 9 and to the human fetus as early as week 5 and there are biologically relevant levels of fT4 in the first trimester [Calvo et al., 2002; Contempré et al., 1993; Woods et al., 1984]. The thyroid receptors appear in the brain around gestation day (GD) 14 in rats and week 10 in humans [Bernal & Pekonen, 1984; Ferreiro et al., 1988]. However, as fetal thyroid function does not initiate until GD 17 in rats and week 18 in humans, thyroid hormones are supplied via the maternal thyroid gland and transported across the placenta to the fetal circulation (fig. 5) [Calvo et al., 2002; Fisher et al., 1976; Patel et al., 2011b]. Many transporters may be implicated in placental transfer, including TTR [Landers & Richard, 2017; Patel et al., 2011a]. In the fetal circulation TTR distributes TH to tissues, with the fetal brain relying exclusively on maternal T4 and not T3 [Calvo et al., 1990; Grijota-Martínez et al., 2011]. T4 is proposed to enter the brain via two mechanisms: across the blood-brain barrier by MCT8 uptake into endothelial cells and then into astrocytes via organic anion transporter 1C1 (OATP1C1), and across the blood-cerebrospinal fluid barriers via MCT8 and OATP1C1 in choroid plexus epithelial cells, followed by intracellular complexation with TTR and export into the cerebrospinal fluid where TTR distributes T4 to the brain [Chanoine et al., 1992; Landers & Richard, 2017; Wirth et al., 2014]. In astrocytes T4 is deiodinated by D2 and T3 is sent to neurons through MCT8 for receptor binding, with subsequent degradation performed by D3 [Freitas et al., 2010; Zoeller, 2010]. It has also been suggested that T4 exerts direct action on TRα1

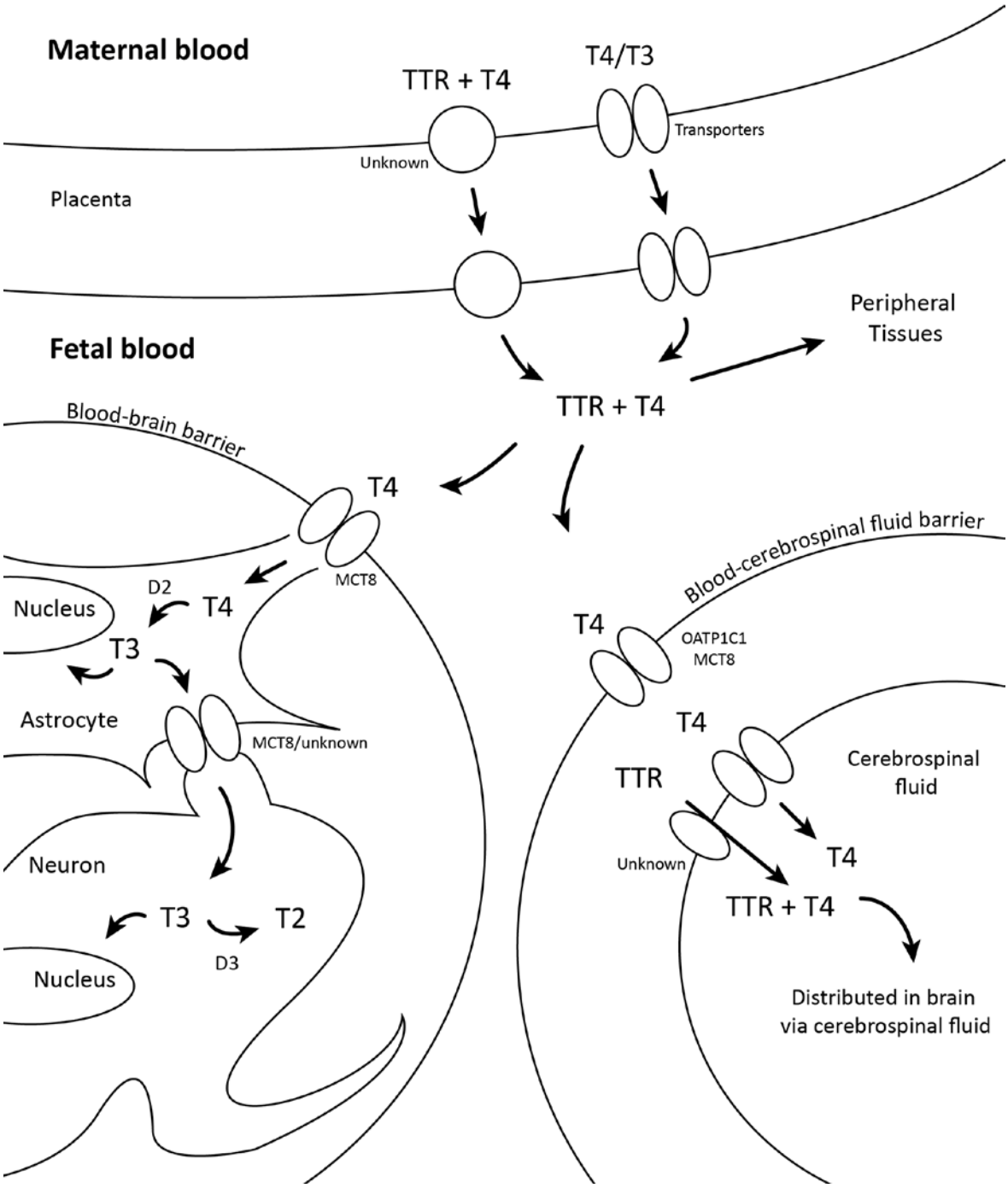

Figure 5 | Transport of thyroid hormones from the maternal circulation to the fetal brain. See text for details. T4: thyroxine, T3: tri-iodothyronine, D2 and D3: deiodinase type 2 and 3, MCT8: Monocarboxylate transporter 8, OATP: organic anion transporting polypeptide. TTR: transthyretin.

[Schroeder & Privalsky, 2014].
This intricate system of distributors, transporters and metabolizing enzymes allows for careful spatiotemporal timing of thyroid hormone action and some compensatory capacity to ensure important hormone action in times of insufficiency or excess [Morreale de Escobar et al., 1988]. Nevertheless, there are plenty of examples of insufficient compensation in both the fetal and neonatal brain resulting in irreversible effects on brain development in humans [Berbel et al., 2009; Haddow et al., 1999; Henrichs et al., 2010; Korevaar et al., 2016] and rats [Ausó et al., 2004; Gilbert et al., 2014; Lavado-autric et al., 2003; Sharlin et al., 2010; Shibutani et al., 2009].

2.3.4 The rat as model in thyroid hormone disruptor research

The thyroid hormone system is highly conserved across species and in mammals the molecular similarities are significant. The ontogenesis of the system is similar in rats and humans, albeit temporal differences as the final stages of maturation happen postnatally in rats (Fischer 1976). Nevertheless, rats and humans share roughly the same thyroid hormone system, but with some distinct differences in especially metabolic rates. The rat thyroid hormone system runs at a faster pace with a shorter thyroid hormone half-life, than the human system. Consequently, the adult rat appears to have impaired compensatory capabilities, in response to thyroid hormone disruptors compared to adult humans [Fisher et al., 2012; McClain, 1989]. However, during pregnancy, the maternal human thyroid system, supplying the fetus, is already vulnerable and hormone production increased to meet increased demands, for some beyond capacity (see section 2.3.6). Also neonates are uniquely vulnerable with high hormone turnover and no stored iodine or hormone [Ginsberg et al., 2007; Glinoer, 1997]. At the same time, just slightly inadequate thyroid function during the fetal and neonatal periods may have consequences for child brain development (See 2.4.2 and 6.4). This suggests that a potential increased relative sensitivity of the rat thyroid system could make the rat a good model for human thyroid function during development. On the other hand, with regards to brain development, the rat brain cannot model the full spectrum of complex human neurobehavior and cognition [Berbel et al., 2014].

The relative distribution between serum distributor proteins is different between rats and humans with TTR being predominant in the rat and TBG mostly being present during rat development. In humans TBG is the most abundant, binding 70% of thyroid hormones in the serum. However, 15% of the hormones are bound to TTR, and this fraction appear to

be the biologically active as the affinity to TTR is just enough to protect thyroid hormones from degradation, yet low enough to release thyroid hormones at target cells [Alshehri et al., 2015]. Thus underscoring the similar hormone systems in the two species.

The differences between human and rat thyroid systems complicates the risk assessment process and warrants further studies. At the same time, thyroid disorders are one of the most frequent human endocrine disorders with 11% of the European population affected [Madariaga et al., 2014] and a rising frequency of thyroid cancer [Enewold et al., 2009], suggesting that the human thyroid system is not infallible.

2.3.5 Thyroid hormone balance in the rat during development

The origin of thyroid hormone supply varies throughout fetal development and early postnatal life. The rat fetal thyroid gland is not fully functional until GD 21 and hence T4 is transferred across the placenta from the dam until late gestation where the fetal gland gradually takes over production. Towards the end of gestation dam TSH is suppressed and circulating TH falls steadily. Around birth ca 17% of the thyroid hormones in the neonate are of maternal origin [Morreale de Escobar et al., 1990]. After birth rising TSH levels stimulate T4 production to a steady increase from around PND 5 towards PND 17 where it reaches a maximum, before a gradual decline towards the adult equilibria reached around PND 35 [Dussault & Labrie, 1975; Fisher et al., 1976; Howdeshell, 2002].

2.3.6 Thyroid hormone economy during pregnancy and breastfeeding

In humans, pregnancy brings on physiological changes that include changes to the thyroid hormone system and increase the demand of hormone from the gland. Both total and fT4 rise during the 1st trimester, approximating steady state conditions from gestational week 12 until term. Throughout pregnancy circulating levels of T3 do not seem to change much or they follow the fT4 levels with a modest rise early in pregnancy and later a small reduction [Patel et al., 2011a]. The changing hormone levels fall within normal reference intervals but it complicates evaluation of thyroid hormone balance because different stages

of pregnancy has their individual reference intervals that varies with the laboratory performing the measurements [Lazarus & Mestman, 2012].

These more modest changes to the circulating hormone levels masks more marked physiological changes. From early in pregnancy plasma TBG rise due to increased synthesis and a stabilizing effect of estrogen, leading to increased T4 levels [Brent, 1997]. While T4 levels rise during the first trimester, it suppresses TSH levels, but human chorionic gonadotropin (hCG) seems to replace TSHs thyroid stimulating role during these first 12 weeks, and its works to further activate the gland [Mestman, 1998]. Later in gestation these stimulatory events are followed up by changes in the peripheral metabolism of thyroid hormones, especially in the placenta [Lazarus & Mestman, 2012]. Additionally, pregnancy brings plasma expansion and increases renal excretion of iodine, potentially reducing the iodine available to the thyroid gland. All of these factors work together for a net result of increased demand of thyroid hormone [Alexander et al., 2004]. Healthy thyroid systems can meet these requirements, but impaired systems may not be able to, and the combination of pregnancy and conditions as autoimmune thyroid disorders and selenium, iron and iodine deficiency may decrease thyroid capacity and interact with pregnancy to cause thyroid insufficiency [Thilly et al., 1992].

2.4 The importance of thyroid hormones during brain development

For thyroid hormone disruptors the primary area of concern is potential effects on neurodevelopment. Here, the evidence for associations between low thyroid hormone function and effects on neurodevelopment will be reviewed.

2.4.1 Thyroid hormones and brain development in animal studies

Thyroid hormones are essential for both fetal and postnatal brain development and their functions during brain development has been extensively reviewed, including: [Bernal, 2005, 2015, 2017; Demeneix, 2014; Dussault & Ruel, 1987; Gilbert & Zoeller, 2011; Howdeshell, 2002; Moog et al., 2017; Morreale de Escobar et al., 2004; Porterfield & Hendrich, 1993; Williams, 2008; Zoeller & Rovet, 2004].

In the rat, thyroid hormones are necessary for neurogenesis from around GD 12 in the developing brain. While some areas primarily have neurogenesis during gestation it stretches at least until weaning in the cerebellum, hippocampus and olfactory bulb [Howdeshell, 2002; Mohan et al., 2012]. Thyroid hormones influence migration so that insufficiency results in densely packed neurons and migration defects [Gilbert et al., 2014; Mohan et al., 2012; Shibutani et al., 2009]. Later in gestation it is followed by synaptogenesis also influences by thyroid hormones. In hypothyroidism, neural connections are impaired by decreased axonal growth and branching of neurons. The circuitry is further impaired by postnatal effects on oligodendrocyte differentiation and myelination with the developmental hypothyroid brain lacking myelination and white matter volume [Berbel et al., 1994; Powell et al., 2012; Sharlin et al., 2008]. In the cerebellar cortex lack of thyroid hormone results in maturation defects of the purkinje cells with decreased arborization and a permanent impairment of neuronal connectivity [Legrand, 1979].

The effects of developmental hypothyroxinemia and hypothyroidism on brain development are well established. However, the majority of studies relied on animal models of severe thyroid hormone deficiency. These studies used doses of antithyroid drugs as 10 ppm

propylthiouracil (PTU)/200 ppm methimazole (MMI) (and above) in drinking water, thyroidectomy or radioiodine treatment. They report a range of effects on the developing brain at the highest doses; structure, morphology, function, and behavior including learning and memory are all affected [Akaike et al., 1991; Berbel et al., 1994; Dussault & Ruel, 1987; Gilbert & Zoeller, 2011; Goldey et al., 1995; Iniguez et al., 1996; S. Y. Kim et al., 1998; Ruiz-marcos et al., 1979]. Models of milder and transient forms of thyroid hormone insufficiency are sparser but effects such as migration errors, synaptic transmission, and plasticity, and subtle impairments in learning has been found [Gilbert, 2011; Gilbert et al., 2013, 2016, 2017; Hassan et al., 2017; Mohan et al., 2012; Powell et al., 2012]. Also, transient (3 days) exposure to MMI has resulted in a significant proportion of, primarily, neurons in aberrant and inappropriate places matched to their birth date, this deranged migration resulted in blurred cell layers of both cortex and hippocampus. Additionally, the migration was correlated to wild runs and audiogenic seizures in the adult offspring and it could be rescued with concurrent T4 treatment [Ausó et al., 2004; Lavado-autric et al., 2003].

Table 1. Thyroid conditions

Serum	fT4	TSH
	Reference range of population	
Overt hypothyroidism	fT4 < 0th percentile	elevated
Subclinical hypothyroidism	normal	elevated
Hypothyroxinemia	0th percentile < fT4 < 10^{th} percentile	normal

2.4.2 Thyroid hormones and brain development in humans

The importance of thyroid hormones for human development has been established through studies of congenital hypothyroidism and in areas of endemic iodine deficiency. Congenital hypothyroidism is a condition in which the neonate cannot produce thyroid hormones of their own, they are born with very low levels of thyroid hormones but appear normal due to a sufficient supply of thyroid hormones from the mother during pregnancy. However, without treatment the continued development of the children is severely inhibited and they suffer from severe mental retardation, spasticity, incoordination and hearing loss [Delange, 1994; Gilbert & Zoeller, 2011]. The symptoms can largely be avoided with early and sufficient treatment, but suboptimal neurodevelopment can still be observed [Zoeller & Rovet, 2004].

Severe iodine deficiency during fetal development has even more detrimental consequences as motor milestone retardation, deaf-mutism, squint and mental deficiency, but endemic cretinism is preventable with adequate iodine supply before conception [Pharoah et al., 1971].
While the effects of pronounced thyroid hormone deficiency during development are detrimental, milder forms of thyroid hormone deficiency at different stages of fetal and postnatal brain development may result in many less severe effects that have been studied in association studies usually correlating maternal hypothyroidism or hypothyroxinemia (Table 1) with child cognition and behavior.

IQ scores is a common end-point in thyroid studies and effects are often reported. E.B. Man was the first to study the effect of maternal hypothyroxinemia on child brain development. In one trial with multiple follow-ups they showed that inadequately treated

hypothyroxinemia was correlated to decreased child IQ at age 7 and conversely, that adequately treated hypothyroxinemia was correlated to increased IQ [Man, 1972; Man & Serunian, 1976]. Hypothyroidism during pregnancy resulted on average in a loss of 7 IQ points at age 7-9 with 19% of the children falling below 85 IQ points (5% in the matched controls) and effects within all 5 parameters of the neuropsychological test; intelligence, attention, language and school performance [Haddow et al., 1999]. Some of the women were treated for hypothyroidism during pregnancy and despite serum levels of TSH not returning to normal, it improved the outcome in the children. Henrichs et al., (2010) studied both mild (fT4<10^{th} percentile) and severe (fT4<5^{th} percentile) maternal hypothyroxinemia. Mild hypothyroxinemia was associated with an increased risk of expressive language delay in the children at age 18 months and severe hypothyroxinemia with verbal and nonverbal cognitive delay at 36 months [Henrichs et al., 2010]. Ghassabian et al. (2014) showed a mean reduction in IQ of 4.3 points due to maternal fT4<5^{th} percentile with normal TSH but found no correlations between brain morphology and volume measures in a potentially underpowered sub-analysis [Ghassabian et al., 2014]. Korevaar found a similar reduction in IQ between 1.4-3.8 points associated with both low and high maternal fT4 levels, and in addition an u-shaped correlation to grey matter and cortex volume [Korevaar et al., 2016].

Autism, Attention Deficit Hyperactivity Disorder (ADHD), and schizophrenia have also been linked to low thyroid hormone during development. Maternal hypothyroxinemia with iodine supplementation from 12-14 weeks of gestation or not at all was associated with lower developmental quotient in the children at 18 months compared to the quotient in children whose mothers were euthyroid and who had received iodine supplementation from week 4-6 [Berbel et al., 2009]. Additionally, there was effects on gross and fine motor skills and socialization but not on language; a pattern suggesting predisposition to development of autism [Berbel et al., 2009]. In a nationwide Danish cohort study hypothyroidism during pregnancy was associated with diagnosis of a child with autism spectrum disorders [Andersen et al., 2014], and the Dutch generation R study also found that severe maternal hypothyroxinemia increased the odds ratio with about 4 for having a child that was scored as “probably autistic” [Román et al., 2013]. Vermiglio et al. (2004) studied ADHD up until age 8-10 in a moderately iodine insufficient area compared to a marginally iodine sufficient area [Vermiglio et al., 2004]. They found that 11 out of 16 children from the iodine deficient area had ADHD (vs 0 out of 11 in controls) and that IQ in the iodine insufficiency area was 92 vs 110 in controls. In addition, overall ADHD prevalence in the iodine deficient area was higher and fT4 levels below normal [Vermiglio et al., 2004]. A less pronounced effect, but still significant, was found on ADHD in a study of mild maternal hypothyroxinemia and ADHD at age 8 [Modesto et al., 2015], whereas only maternal TSH, and girls ADHD was associated in another study [Päkkilä et al., 2014].

Recently, another study found that maternal hypothyroxinemia was associated with later life development of schizophrenia [Gyllenberg et al., 2016].

Overall, studies show concordance between low thyroid hormones during development and impaired neurobehavioral development of the child [Berbel et al., 2009; Gyllenberg et al., 2016; Haddow et al., 1999; Henrichs et al., 2010; Korevaar et al., 2016; Modesto et al., 2015; Pop et al., 2003] and this is supported by recent systematic reviews and meta-analysis [Fetene et al., 2017; Thompson et al., 2018]. However, to date, the two randomized controlled trials investigating the effect of maternal levothyroxine treatment on child neurobehavioral development have not found an effect [Casey et al., 2017; Lazarus et al., 2012] and another study raises concerns for adverse effects arising from overtreatment with levothyroxine resulting in high-normal fT4 levels [Korevaar et al., 2016]. This indicates that our understanding of the complex relationships between thyroid function and brain development is limited and that further studies are needed. These inconsistencies between adverse effects of inadequate T4 supply, yet lack of apparent effect of treatment, could arise due to underlying confounding factors and relationships causing or obscuring effects in studies of thyroid function. The epidemiological studies point to several areas of concern for further studies, also for their potential influence on the results of studies investigating associations between chemical exposures and thyroid function.

2.4.3 Confounding factors in epidemiologic studies of thyroid function and neurobehavioral development

Epidemiological study designs can influence the outcome. Study population, hormone limits and values, reference intervals, and time of hormone assessment during gestation are all parameters that can influence the results. The definition of impaired thyroid function has implications for the outcome; more effects were present in “severe” hypothyroxinemia defined as fT4<5^{th} percentile and fewer effects in “mild” hypothyroxinemia 5^{th} percentile<fT4<10^{th} percentile [Henrichs et al., 2010]. Outcomes have been different depending on whether data was analyzed as the hypothyroxinemic group compared to the rest of the population [Ghassabian et al., 2014] or by linear regression [Korevaar et al., 2016].
As described previously, thyroid function changes over the course of pregnancy and associations to neurobehavior may vary according to which point in time TH measure-

Iodine and thyroid function

Iodine deficiency is the most common dietary deficiency that can give rise to neurodevelopmental problems, with almost 2 billion worldwide at risk, including >40% of the European population[1].

The iodine deficiency disorders (IDDs) span the endemic and severe iodine deficiency giving rise to endemic cretinism with severe mental retardation, deaf-mutism and motor spasticity but also the milder forms of iodine deficiency which gives rise to neurological impairments on a population scale. Due to these effects, the importance of adequate supplies of iodine for optimal neurodevelopment cannot be understated[2].

Iodine status of a population is tricky to assess as both inter-individual and intra-individual variation in urinary iodine concentration is high, necessitating measurements from ca 10 days to determine iodine status of an individual with ca. 20% precision[3].

Mild and moderate iodine insufficiency may result in higher T3 and lower T4 levels but it is an insensitive measure as hormone levels may be within reference intervals and they have a large overlap with iodine sufficient populations[4].

[1][Andersson et al., 2012; Bath et al., 2013; Bath & Rayman, 2013; WHO, 2007]
[2][Pharoah et al., 1971; Zimmermann, 2012]
[3][König et al., 2011]
[4][Berg et al., 2017; Brucker-Davis et al., 2012; Glinoer, 1997; WHO, 2007]

ments are performed. Pop et al., (2003) found that Maternal fT4<10th percentile at week 12 of gestation was associated with impaired motor and mental development of the child at age 1 and 2. Interestingly, this effect was attenuated by higher fT4 at week 24 and 32 and increased by lower values later in pregnancy. Yet, lower fT4 at week 24 and 32 had no effects in the control population [Pop et al., 2003]. These results suggest that effects observed in studies with only a single thyroid function determination during gestation may under or overestimate the effects depending on the subject's thyroid status over the course of pregnancy and that time of assessment is critical.

Studies usually assess a child's postnatal neurobehavioral development and the relationship to thyroid hormones during development. However, a range of factors, in addition to thyroid hormone, can impact on a child's neurobehavioral development and may influence it from birth and until assessment. These include parental education level, household income, marital status, urbanization at birth, smoking, siblings, ethnicity, birth weight, sex,

gestational time, breastfeeding, hospitalizations, environmental and psychosocial factors [Ghassabian et al., 2014; Gyllenberg et al., 2016; Haddow et al., 1999; Korevaar et al., 2016; Pop et al., 1995, 2003]. In addition to these factors related to the individual child and study population, variability between studies also arise from the applied assessment: type of test, test performer, age of child at assessment, and the measurement of different endpoints may all give rise to different outcomes.

Variation also arises from the physiological conditions of thyroid function. Sometimes there are associations between effects and TSH irrespective of fT4 [Ghassabian et al., 2014; Päkkilä et al., 2014] and sometimes not [Gyllenberg et al., 2016; Henrichs et al., 2010; Korevaar et al., 2016]. Studies also suggest the existence of a subpopulation of TPO-antibody (ab) positive individuals which are uniquely sensitive to disturbances of the thyroid hormones [Brown et al., 2015; Nøhr et al., 2000; Pop et al., 1995, 1999; Webster et al., 2014]. Iron deficiency can interfere with the iron dependent TPO enzyme and selenium deficiency with the iodothyronine deiodinases both potentially exacerbating effects of thyroid hormone disturbances and levels [Bastian et al., 2014; Köhrle, 2015; Preston et al., 2018; Webster et al., 2014].

Last is the influence of individual and population iodine status. Production of thyroid hormones is dependent upon iodine and milder forms of deficiency has modified the outcome of studies investigating thyroid hormone deficiency and neurobehavioral development, with or without a measurable effect on thyroid status [Bath & Rayman, 2012; Berbel et al., 2009; Lazarus et al., 2012; Taylor et al., 2014; Vermiglio et al., 2004]. Thus, controlling for iodine status in studies of thyroid function is important, albeit complicated [Bath & Rayman, 2012; König et al., 2011; Lazarus et al., 2012](see also box “Iodine and thyroid function).

2.5 Perfluorinated compounds and thyroid status

Exposure to perfluorinated compounds consistently affects circulating levels of thyroid hormones in animal studies. However, the potential effects of PFHxS *in vivo* was unclear, and thus investigated in chapters 3 and 4. Here, the potential mode of actions of PFHxS on the thyroid system of experimental animals and humans will be reviewed.

2.5.1 Mode of action of PFAS in the thyroid hormone system

The Hypothalamic-Pituitary-Thyroid axis

The HPT axis contains the central compensatory feedback loops of the thyroid hormone system (section 2.3.1) that maintain TH levels within narrow concentration levels. Hence, traditionally, we expect the HPT axis to increase TSH production when serum T4 is decreased [Bansal et al., 2014; Goldey et al., 1995; Klaassen & Hood, 2001; Kortenkamp et al., 2017; Yu, Liu, & Jin, 2009]. However, some environmental chemicals seem to circumvent the feedback loops and decrease hormone levels without increasing TSH levels and activating the compensatory responses of the endocrine system (chapter 4) [Bansal et al., 2014; Goldey et al., 1995; Klaassen & Hood, 2001; Kortenkamp et al., 2017; Yu, Liu, & Jin, 2009]. The underlying physiology and consequences of this atypical response is still elusive [Kortenkamp et al., 2017].

A similar pattern of effects is seen after PFOS exposure [Bjork et al., 2008; Chang et al., 2008, 2009; Lau et al., 2003; Luebker et al., 2005; Thibodeaux et al., 2003]. Direct damage of the HPT axis is not the cause as co-exposure to PTU induced the expected compensatory increase in TSH levels [Chang et al., 2008]. Another study, where PFOS was administered in drinking water to male rats for 91 days, found no significant effect on TSH, although levels were nominally doubled (0.72±0.30 IU/L in controls versus 1.62±0.67 IU/L in 15 mg/L group) while serum T4 was decreased to 20% of control levels [Yu, Liu, & Jin, 2009]. However, in contrast, the Butenhoff et al., (2009) study indicated a potential activation of the axis in the males receiving the highest dose as they found histopathological changes in the thyroids of the male rats [Butenhoff, Chang, et al., 2009]. Possibly, due to a high internal exposure of male rats compared to females [Butenhoff, Chang, et al.,

2009; S.-J. Kim et al., 2016; Ramhøj et al., 2018; Sundström et al., 2012]. Later, Chang et al., 2018 reported no effects on histopathology or TSH in mice receiving a rather low exposure to PFHxS [S. Chang et al., 2018]. The effect of PFHxS on the HPT axis is further investigated in chapter 4.

Thyroid hormone distributor proteins

One of the classical targets of thyroid hormone disrupting chemicals are TTR, TBG and albumin; three plasma proteins involved in distributing thyroid hormones to target tissues. These proteins have a high affinity to thyroid hormones, but can also bind to a range of chemicals, including PFHxS and other PFASs. For instance, PFHxS can bind to both TTR and albumin, but not TBG [Kerstner-Wood et al., 2003; Ren et al., 2016; Weiss et al., 2009]. Amongst PFASs, PFHxS and PFOS have high affinities to TTR, albeit still 4-20 times lower than the affinity for T4 [Ren et al., 2016; Weiss et al., 2009]. Despite the limited affinity relative to T4, both PFOS and PFOA have theoretically been shown capable of binding to almost all of the available TTR in occupationally exposed workers (5.3µM chemical-bound TTR vs. 5.4µM TTR in normal human serum) [Ren et al., 2016]. Taking mixture effects from all chemicals binding to TTR [Crofton et al., 2005; Flippin et al., 2009; Weiss et al., 2009] into account it shows the potential for effects in humans.

The precise implications of chemicals binding to TH distributor proteins remains uncertain, but it can be speculated that the end result will be less TH available to target tissues, including the developing fetal brain. Furthermore, TTR could directly transport chemicals from the maternal circulation, across the placenta to the fetal compartment and into the fetal brain where PFOS have been found [Alshehri et al., 2015; Chang et al., 2009; Chanoine et al., 1992].

Distributor proteins also act to shield TH from hepatic elimination, thereby prolonging TH half-life. Displacement of TH from distributor proteins could increase biliary excretion leading to decreased circulating TH levels, as for instance is the case for PFOS. Within 2-6 h of PFOS exposure there are transiently increased free T4 levels inducing expression of the thyroid hormone responsive gene malic enzyme and reducing TSH levels. Concurrently, an upregulation of the T4 glucuronidation enzyme UGT1A1 messenger ribonucleic acid (mRNA) increases turnover and loss of T4 resulting in persistently reduced total T4 levels [Chang et al., 2008; Yu, Liu, & Jin, 2009]. Hence, evidence supports the view that reduced serum protein binding of T4 increases hepatic enzyme activity and elimination, and that fT4 drives TH responsive gene expression. The similarities between PFHxS and PFOS and the affinity to TTR suggest that also PFHxS could act through this mechanism *in vivo*.

Liver metabolism

Another classical target of thyroid hormone disrupting chemicals is the liver enzymes that eliminate thyroid hormones by glucuronidation and sulfation. The expression and activity of UGTs and SULTs can be induced by both drugs and environmental chemicals resulting in loss of TH. Indeed, the liver is a target organ of both PFHxS and PFOS. Findings include upregulation of UGT1A1 mRNA along with reduced liver D1 and activation of both peroxisome proliferator-activated receptor (PPAR) α, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) pathways [Bjork et al., 2008; Butenhoff, Ehresman, et al., 2009; S. Chang et al., 2018; Chang et al., 2009; H. Dong et al., 2016; Rosen et al., 2017; Yu, Liu, & Jin, 2009; Yu, Liu, Jin, et al., 2009]. However, the exact effects on UGT-activity and mRNA are unknown for PFHxS and it should be noted that, in general, effects on mRNA levels are not always reflected in enzyme activities and vice versa [Bárez-López et al., 2017; Glanemann et al., 2003; Ohtsuki et al., 2011]. Furthermore, it appears that the effects on TH are evident at doses lower than the doses at which effects in the liver are detected (also see chapter 4). This shows either a lack of sensitivity of applied liver investigations or that increased hepatic elimination of thyroid hormone is not the primary mechanism of PFOS/PFHxS mediated thyroid hormone reductions.

2.5.2 Human epidemiology studies of PFAS and thyroid status

Multiple epidemiological studies have investigated the relationship between exposures to various PFAS and thyroid hormones in different populations. In many studies, the adjustment for confounders attenuates associations, sometimes allegedly due to correlations between exposures to the different compounds. Yet, most studies are not designed to assess joint exposures to PFAS or effects are not significant in a model of all exposures [Berg et al., 2015; S. Kim et al., 2011]. The studies are of heterogeneous design, as are the results with significant effects associated to PFHxS: increased fT4 and total T4 in adults [Liu et al., 2018], increased T4 in adults [Jain, 2013], increased fT4 in adults [Seo et al., 2018], increased T3 in newborn girls [Shah-Kulkarni et al., 2016], increased T3 in 60-80 year old females, increased T4 in 20-40 year females [Lewis et al., 2015], reduced fT4 in TPO-ab positive pregnant women [Webster et al., 2014], inversely associated to maternal fT4 [Preston et al., 2018], negatively to fT4 [Webster et al., 2016], decreased T4 in male adolescents [Lewis et al., 2015], and decreased T4 in 60-80 year females [Lewis et al.,

2015]. There is also studies [Berg et al., 2015; Chan et al., 2011; S. Kim et al., 2011; Wang et al., 2013; Yang et al., 2016] and endpoints in the above mentioned studies that show no significant correlations to PFHxS. Identification of some effects seems to depend and vary according to exposure level, sex, age, TPO-ab status, iodine status, smoking, body mass index (BMI), mother versus fetal, and hormones assessed. The heterogeneity of results is not surprising if comparing to epidemiological studies of other thyroid disrupting compounds [Boas et al., 2009, 2012; Ghassabian & Trasande, 2018; Mughal et al., 2018], but suggest some of the challenges associated with epidemiological studies of both thyroid status and chemical exposures. Despite this, recent reviews and meta-analysis conclude that, overall, there is evidence suggesting that PFAS modifies thyroid hormone homeostasis (primarily hypothyroidism) in especially pregnant women [Ballesteros et al., 2017; Coperchini et al., 2017; Lee & Choi, 2017] whereas others did not find that in a subanalysis of pregnant women [M. J. Kim et al., 2018]. Specifically Ballesteros et al., (2017) found correlations between PFHxS and TSH in maternal blood and Kim et al., (2018) that PFHxS is negatively correlated to T4 in the general population [Ballesteros et al., 2017; M. J. Kim et al., 2018].

2.6 Perfluorinated compounds and the develoing immune system

Allergic, autoimmune and inflammatory diseases are reaching epidemic proportions in these years [Minihane et al., 2015; H. Okada et al., 2010; Patterson et al., 2009; Pearce et al., 2007]. Mutual for them all are a dysregulation of the immune system, leading to hyperactivation, inadequate activation, or even activation towards the organisms own components. The resulting disorders may be fatal and can have serious impacts on the quality of life of the individual and presents a considerable economic burden on societies [Global Asthma Network, 2014; NIH, 2005; AARDA, 2011]. The prevalence has been increasing in a pace that indicates environmental causes and it is possible that early life insults on the developing immune system [Dietert, 2008; Pollard et al., 2010; Tonk et al., 2012; WHO/UNEP, 2013] can result in altered developmental programming causing impaired immunological function later in life [Dietert, 2008; Dietert & Zelikoff, 2008].

Within risk assessment and toxicology the immune system presents a special case due to its presence in virtually every tissue and to its functional nature. Consequently, it can be assumed that every chemical exposure will result in exposure of some components of the immune system, but also, that it is crucial to test whether the function of the immune system, is altered by an exposure [IPCS, 2012]. This, in combination with an exposure taking place during development of the immune system, presents a special challenge to toxicology [Dietert, 2008]. It is necessary to consider both the acute effect of an exposure during development, as well as any latent future effects on the different components and functions of the immune system [Tonk et al., 2010].

2.6.1 Perfluorinated compounds and immunotoxicity

In 2012, Dewitt and collegues concluded that exposure to perfluorinated compounds can adversely affect immune function [DeWitt et al., 2012]. This was based on observations in turtles and dolphins, *in vitro* to *in vivo* data correlations, and overall concordance between results from animal studies. Since then, especially more human studies have been published and in 2016 a 3M (previous producer of PFOS and PFOA) sponsored review concluded that “epidemiological evidence does not establish a causal effect of PFOA or PFOS on immune conditions in humans” [E. T. Chang et al., 2016]. While the National

Toxicology Program (NTP) concluded that exposure to both PFOA and PFOS are "presumed to be an immune hazard to humans" [NTP, 2016].

Studies of PFOA and PFOS immunotoxicity indicate that they share some, but not all, properties and show differential effects and modes of action *in vivo* and *in vitro* [Corsini et al., 2011, 2012, 2014; Midgett et al., 2015; Mogensen, Grandjean, Nielsen, et al., 2015; Qazi et al., 2009]. PFHxS may possess similar properties [Rosen et al., 2017; Wolf et al., 2014]. Some of the hallmark effects are altered antibody production [Grandjean et al., 2012; Grandjean, Heilmann, Weihe, Nielsen, Mogensen, & Budtz-Jørgensen, 2017; Grandjean, Heilmann, Weihe, Nielsen, Mogensen, Timmermann, et al., 2017; Grandjean & Budtz-Jørgensen, 2013; Granum et al., 2013; Keil et al., 2008; Kielsen et al., 2016; Looker et al., 2014; Mogensen, Grandjean, Heilmann, et al., 2015; Peden-Adams et al., 2008] and skewing of the immune system towards a T helper cell type 2 (Th2) response [G.-H. Dong et al., 2011, 2013; Keil et al., 2007; Midgett et al., 2015; Timmermann et al., 2014, 2015, 2017; Zheng et al., 2011; Zhou et al., 2017; Zhu et al., 2016]. Decreased Immunoglobulin M (IgM) production was found in male mice after exposures down to 1.66 µg PFOS/kg/day for 28 days, bringing the mice's serum levels within the range of human serum levels [Peden-Adams et al., 2008]. Gestational exposure (GD 1-17) of mice to PFOS resulted in decreased natural killer (NK) cell activity and decreased IgM response to sheep red blood cells at 8 weeks of age [Keil et al., 2008]. Corsini et al 2014 suggested some PFAS mediated immunity to be through PPARs and the nuclear factor-κB (NF-κB) pathway. This has been confirmed in humans where the PPARδ and NF-κB pathways were enriched in a transcriptomics study linking prenatal PFAS (PFOS, PFOA, perfluorononanoic acid (PFNA), and PFHxS) exposure with childhood immunological events [Pennings et al., 2016]. Overall, immunological and developmental pathways were affected by both prenatal PFAS exposure and occurrences of the common cold and rubella antibody titers in early childhood. In addition the specific linkage of PPARδ and NF-κB provides a mechanistic explanation for the observed immunotoxic effects, also in humans, and strengthens the notion that also prenatal PFAS exposure affects human immunological health [Pennings et al., 2016].

In humans there may be consistent correlations between PFAS and immune effects overall. However, what specific compounds that show statistically significant effects after adjustment for confounders differ, including exposure to the other perfluorinated compounds usually correlated to each other. Mogensen et al., 2015 performed a joint analysis on PFOA, PFOS and PFHxS exposure in 7-year old children and found a doubling of serum PFAS resulted in a 54% decrease in protective antibodies after vaccination towards difteria and tetanus [Mogensen, Grandjean, Heilmann, et al., 2015]. The authors considered these strongly decreased antibody concentrations to reflect an immunological deficit which

should be regarded as adverse and their calculations showed that current exposure levels to PFOA and PFOS can cause impaired immune function in Faroese children [Grandjean & Budtz-Jørgensen, 2013; Mogensen, Grandjean, Heilmann, et al., 2015]. When looking at the compounds individually, it appears that PFHxS had the biggest effects on tetanus antibodies compared to diphtheria [Mogensen, Grandjean, Heilmann, et al., 2015].
Furthermore, exposure to PFHxS has been statistically significantly associated to self-reported food allergies in adolescents [Buser & Scinicariello, 2016], childhood atopic dermatitis in girls [Chen et al., 2018], asthma and asthma biomarkers [G.-H. Dong et al., 2013; Zhou et al., 2017; Zhu et al., 2016], asthma in 5-year old measles mumps and rubella (MMR)-unvaccinated children [Timmermann et al., 2017], total infectious diseases in girls [Goudarzi et al., 2017], common cold [Granum et al., 2013], gastroenteritis [Granum et al., 2013], anti-tetanus antibodies [Grandjean, Heilmann, Weihe, Nielsen, Mogensen, Timmermann, et al., 2017; Mogensen, Grandjean, Heilmann, et al., 2015], anti-diphtheria antibodies [Grandjean, Heilmann, Weihe, Nielsen, Mogensen, Timmermann, et al., 2017; Mogensen, Grandjean, Heilmann, et al., 2015], anti-rubella antibodies [Granum et al., 2013; Stein et al., 2016] but also to less sensitization to cockroach and shrimp [Stein et al., 2016]. Significant finding thus show overall tendencies for increased disease susceptibility, increased asthma and allergic disease and decreased protecting antibodies after vaccinations.
Not surprisingly there were also endpoints in the above mentioned studies that showed no correlations to PFHxS. Other studies found no significant associations between PFHxS exposure and fever [Dalsager et al., 2016], common cold [Impinen et al., 2018], respiratory tract infections at 0-10 years [Impinen et al., 2018], immediate post-booster tetanus and difteria antibodies [Kielsen et al., 2016], eczema and allergic disease [E. Okada et al., 2014; Smit et al., 2015], asthma and allergy biomarker at birth [Ashley-Martin et al., 2015] and asthma in adolescents [Humblet et al., 2014].

The large variability studies and the investigated endpoints make it difficult to draw any conclusions about the specific details of PFAS effects in humans. Critical exposure windows, effect modulation by sex, vaccine-type to be affected and specific effects of single compounds, will all be hard to specify with epidemiological studies alone. The recent study by Timmermann et al., (2017) also clearly shows the importance of correct adjustment for confounders and how important external influences on the dynamic immune system may be. They found a positive correlation between PFAS levels in children and risk of asthma only in the small subgroup of 22 *un*vaccinated children in a cohort study of 559 children [Timmermann et al., 2017]. This association was reversed in MMR-vaccinated children. The authors suggest that PFAS skew the immune system towards a TH2-type response increasing the risk of asthma. However, in the study population the skewing does not happen when a previous MMR vaccination has skewed the immune system towards a

Th1 type response. This potential skewing has been suggested by other recent human studies [Buser & Scinicariello, 2016; G.-H. Dong et al., 2013; Zhou et al., 2017; Zhu et al., 2016], but also not seen in others [E. Okada et al., 2014; Smit et al., 2015; Stein et al., 2016]. It raises further questions about the long term implications of PFAS mediated immunomodulation, not only for decreased resistance against infectious diseases but also for allergic, autoimmune and non-communicable diseases [Dietert, 2014; McFadden et al., 2015].

2.6.2 Testing for developmental immunotoxicity

In a regulatory context, testing for developmental immunotoxicity is only performed as part of the Extended One Generation Reproductive Toxicity Study (EOGRTS), OECD test guideline 443 [OECD, 2011]. Here, the assessment of a cohort 3 for assessment of developmental immunotoxicity is the standard: "decision (…) to omit the developmental (…) immunotoxicity cohort should reflect existing knowledge for the chemical being evaluated, as well as the needs of various regulatory authorities". However, in REACH, cohort 3 is only included if existing knowledge reveal triggers (signs) that indicate a "particular concern" of a "certain level of severity" that a compound has developmental immunotoxic properties [ECHA, 2017c]. Thus, the inclusion of the cohort primarily depends on effects found in less sensitive systems than the developing immune system.
When included, the information requirement for cohort 3 requires assessment of the primary immunoglobulin M response to a T-cell dependent antigen (TDAR), usually using either sheep red blood cells or keyhole limpet hemocyanin (KLH) as antigens (as done in chapter 5. This measure is designed to give "a holistic view of IgM synthesis" and will detect suppression of the immune system, It may also be able to detect stimulation, of the immune response [OECD, 2013]. In addition to this functional immune test, lymphoid organ weights and histopathology, and lymphocyte subset analysis are assessed in the resting immune system of adult F1 offspring. The outcomes of these tests may require further testing of cell mediated immunotoxicity; the delayed type hypersensitivity (DTH) response (as done in chapter 5). If evidence for effects is found, further testing to determine underlying mechanisms can be conducted, these could include immunoglobulin G (IgG) response and regulatory cytokines. In certain cases investigations of resistance to infection, allergic hypersensitivity and autoimmune diseases can be performed in animal disease models [OECD, 2013]. Hence, even though functional immune test are considered the most sensitive and predictive tests [Boverhof et al., 2014; Dietert & Burns-Naas, 2008; Holsapple, 2002; IPCS, 2012; Luster et al., 1992, 1993; OECD, 2013; Tonk et al., 2010]

only the primary IgM response is assessed by default when cohort 3 is included in the EOGRTS study. Despite the TDAR assays being sensitive and predictive of immunotoxicology [Boverhof et al., 2014; Cooper et al., 2006; Luster et al., 1992, 1993] especially related to immunosuppression, it seems that there could be a risk of not detecting developmental immunotoxicity resulting in immune dysfunction and/or disrupted immune regulation. i.e. a risk of missing effects ultimately resulting in increased risks for autoimmune and allergic diseases, viral infections, cancer and misregulated inflammation [Dietert, 2008, 2014; IPCS, 2012] although with increasing chance of detecting such effects when multiple test are combined [Dayan et al., 1998; Dietert & Holsapple, 2007; Holsapple, 2005; Luster et al., 1992, 1993; White et al., 2007].

The implementation of testing for developmental immunotoxicity in each lab should be done with assay optimization for both day and dose of immunizing agent as these may influence results [OECD, 2013; White et al., 2007]. It is also stressed that laboratories should have experience with functional immune testing, be able to deliver accurate and consistent results, and that a positive control study is useful in results interpretation (agents could be e.g. cyclophosphamide, cyclosporine). This seems especially important considering that interindividual variation in TDAR response is a natural feature of the functioning immune system [E. R. Gore et al., 2004; Lebrec et al., 2014]. Examination of validation studies for immunotoxicity testing also show that variation may be high for some assays [Dayan et al., 1998; E. R. Gore et al., 2004; Kawai et al., 2013; Loveless et al., 2007; White et al., 1994]. Furthermore, from comparisons between laboratories with immunosuppressing agents some studies find that although the assays detect immunotoxicity overall, there are still laboratories that do not detect significant effects on some parameters [Dayan et al., 1998; Loveless et al., 2007; Richter-Reichhelm et al., 1995]. White et al., (1994) find more consistency between laboratories on some endpoints. Depending on the agent tested it may be that sheep red blood cells or KLH induces the most sensitive immune response and also that both animal strain and species have an influence on the detected response [Dayan et al., 1998; Kawai et al., 2013; White et al., 2007], also stressing the need for validated test protocols, which can be optimized for reliability [E. R. Gore et al., 2004; Lebrec et al., 2014].

3 Manuscript I

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Research Article

Perfluorohexane Sulfonate (PFHxS) and a Mixture of Endocrine Disrupters Reduce Thyroxine Levels and Cause Antiandrogenic Effects in Rats

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ABSTRACT

The developmental toxicity of perfluorohexane sulfonate (PFHxS) is largely unknown despite widespread environmental contamination and presence in human serum, tissues and milk. To thoroughly investigate PFHxS toxicity in developing rats and to mimic a realistic human exposure situation, we examined a low dose close to human relevant PFHxS exposure, and combined the dose-response studies of PFHxS with a fixed dose of 12 environmentally relevant endocrine disrupting chemicals (EDmix). Two reproductive toxicity studies in time-mated Wistar rats exposed throughout gestation and lactation were performed. Study 1 included control, two doses of PFHxS, and two doses of PFHxS + EDmix ($n = 5$–7). Study 2 included control, 0.05, 5, or 25 mg/kg body weight/day PFHxS, EDmix-only, 0.05, 5, or 25 mg PFHxS/kg plus EDmix ($n = 13$–20). PFHxS caused no overt toxicity in dams and offspring but decreased male pup birth weight and slightly increased liver weights at high doses and in combination with the EDmix. A marked effect on T4 levels was seen in both dams and offspring, with significant reductions from 5 mg/kg/day. The EDmix caused antiandrogenic effects in male offspring, manifested as slight decreases in anogenital distance, increased nipple retention and reductions of the weight of epididymides, ventral prostrate, and vesicular seminalis. PFHxS can induce developmental toxicity and in addition results of the co-exposure studies indicated that PFHxS and the EDmix potentiate the effect of each other on various endpoints, despite their different modes of action. Hence, risk assessment may underestimate toxicity when mixture toxicity and background exposures are not taken into account.

Key words: perfluoronated agents; endocrine disruptors; thyroid; androgens; Reproductive & Developmental Toxicology.

Pre- and postnatal development is under the control of a complex network of tightly regulated processes that ensures health at later adult stages, and disruption of these processes may result in irreversible changes or increased disease susceptibility in adult life. Some of the changes may arise from endocrine disrupting chemicals (EDC), that is, chemicals that can interfere with the hormonal systems and consequently adversely affect numerous organs and organ systems (Bergman *et al.*, 2013). Because human EDC exposure is likely to occur as combined exposures, mixture investigations are relevant to include, when performing animal studies on endocrine disruption (Christiansen *et al.*, 2012; Hass *et al.*, 2012).

Poly- and perfluoroalkyl substances (PFAS) are a diverse group of chemicals that have been used in industrial applications and consumer products, for example, as surfactants since the 1950s (Lindstrom *et al.*, 2011). They are persistent, resist

Table 1. Composition of the Mixture of EDCs (EDmix)[a]

Chemical	CAS Registry Number	Purity	Dose (mg/kg bw/Day)
DBP (dibutyl phthalate)	84-74-2	>99.0%	1.00
DEHP (di-2-ethylhexyl phthalate)	117-81-7	>99.5%	2.00
Vinclozolin	50471-44-8	>99.5%	0.90
Prochloraz	67747-09-5	>98.5%	1.40
Procymidone	32809-16-8	>99.5%	1.50
Linuron	330-55-2	>99.0%	0.06
Epoxyconazole	106325-08-8	>99.0%	1.00
4-MBC (4-methylbenzylidene camphor)	36861-47-9	>98.0%	6.00
OMC (octyl methoxycinnamate)	5466-77-3	>98.0%	12.00
p, p′-DDE (dichlorodiphenyldichloroethylene)	72-55-9	>98.5%	0.10
Bisphenol A	80-05-7	>99.5%	0.15
Butyl paraben	94-26-8	>99.0%	6.00
Total dose			32.11

bw, body weight.
[a]See Axelstad *et al.* (2014) for details.

degradation in the environment, and bioaccumulate in humans (Blum *et al.*, 2015; Scheringer *et al.*, 2014). PFASs can act on various endpoints within the mammalian body, including the metabolic, immune, and endocrine systems (DeWitt, 2015; USDHHS, 2016). There are indications from the industry and biomonitoring studies that the use of the three most commonly found PFAS, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and perfluorohexane sulfonate (PFHxS), has decreased (Kato *et al.*, 2011; Schröter-Kermani *et al.*, 2013; U.S. EPA, 2006). Nonetheless substantial human and environmental exposure to these compounds will continue for some time, due to their long half-lives of 3–-7 years (Olsen *et al.*, 2007). *In vivo* studies have shown that PFOS can cause pup death and thyroid hormone disruption (Lau *et al.*, 2003; Yu *et al.*, 2009a), leading to a classification as a reproductive toxicant category 1B (ECHA, 2018). Epidemiologic studies show inconsistent associations between serum T4 levels and PFOS exposures (Ballesteros *et al.*, 2017; Jain, 2013). PFHxS has recently been classified as a substance of very high concern, due to its very persistent and very bioaccumulative (vPvB) properties, and it is proposed for listing as persistent organic pollutant (POP) according to the Stockholm Convention (ECHA, 2017; UNEP, 2017). In spite of this, surprisingly little is known about the potential toxicity of PFHxS (Danish Ministry of the Environment, 2015; FSANZ, 2016). *In vitro* studies with PFHxS have shown that it can bind to the serum thyroid hormone transport protein transthyretin releasing the hormones to the circulation for hepatic metabolism (Ren *et al.*, 2016; Weiss *et al.*, 2009). As with PFOS, epidemiologic studies investigating PFHxS exposure and human T4 levels find inconsistent associations (Jain, 2013; Wang *et al.*, 2014; Webster *et al.*, 2016).

To our knowledge only one developmental toxicity study with PFHxS has been published (Butenhoff *et al.*, 2009). Toxicity was observed in parental males, with minimal to moderate hypertrophy in liver and thyroid gland appearing at the two highest doses (3 and 10 mg/kg body weight/day). No adverse effects were observed in dams and offspring, however, due to sex differences in elimination of this compound, serum PFHxS were approximately 75% lower in dams than in the parental males receiving the same dose. Additionally dams only showed a minimal PFHxS accumulation in the liver. Hence, we hypothesized lower internal exposures to be the cause of the lack of effects in the dams and offspring.

Due to the evidence of toxicity of PFAS and especially PFOS, the continued human exposure to these compounds and the limited knowledge of developmental toxicity of PFHxS, we decided to conduct a developmental toxicity study of PFHxS in Wistar rats. We focused especially on assessment of endpoints sensitive to endocrine disruption; anogenital distance (AGD), nipple retention (NR), male reproductive organ weights and thyroid hormone levels. Moreover, to investigate internal exposures, PFHxS levels were measured in dam serum. To mimic a more realistic human exposure situation a low dose, closer to human relevant PFHxS exposure, was included, and we also combined the dose-response (Christiansen *et al.*, 2012) studies of PFHxS with a fixed dose of a mixture of 12 environmentally relevant EDCs (EDmix). A similar mixture has previously been investigated by our research group (TotalMix100 in Axelstad *et al.*, 2014; Johansson *et al.*, 2016; Mandrup *et al.*, 2015), and it was administered at a dose reflecting a hundred fold high-end human exposure.

MATERIALS AND METHODS

Chemicals

Perfluorohexane sulfonate (PFHxS) purity >98% was purchased from Sigma-Aldrich (Tridecafluorohexane-1-sulfonic acid potassium salt, CAS-No: 3871-99-6, lot #BCBC3545V, Sigma-Aldrich, Copenhagen, Denmark). The powder was tested for other PFAS impurities by LC-MS, but none were identified (data not shown). Corn oil (Sigma-Aldrich) was used as vehicle for all treatments and dosing in the control group. The mixture of 12 EDCs (EDmix) (chemicals and doses are shown in Table 1) and the rationale behind choosing these compounds and exposure levels has previously been described in detail (Christiansen *et al.*, 2012). Briefly, the mixture consisted of 12 environmental endocrine disruptors, given at a dose reflecting 100 times high end human intakes. For some of the chemicals, the intake estimates were adjusted to reflect the joint exposure to several chemicals of the same class, for example, the dose of epoxiconazole was increased to reflect the exposure to all antiandrogenic azole fungicides. Together the DEHP and DBP doses represented exposure to all antiandrogenic phthalates, whereas for BP and 4-MBC the dose was decreased to adjust for a high intake in specific population groups. However, the TotalMix100 described previously also included acetaminophen (paracetamol) which in the studies presented herein was omitted from the EDmix, resulting in a total mixture dose of 32.11 mg/kg body weight (bw)/day (Table 1).

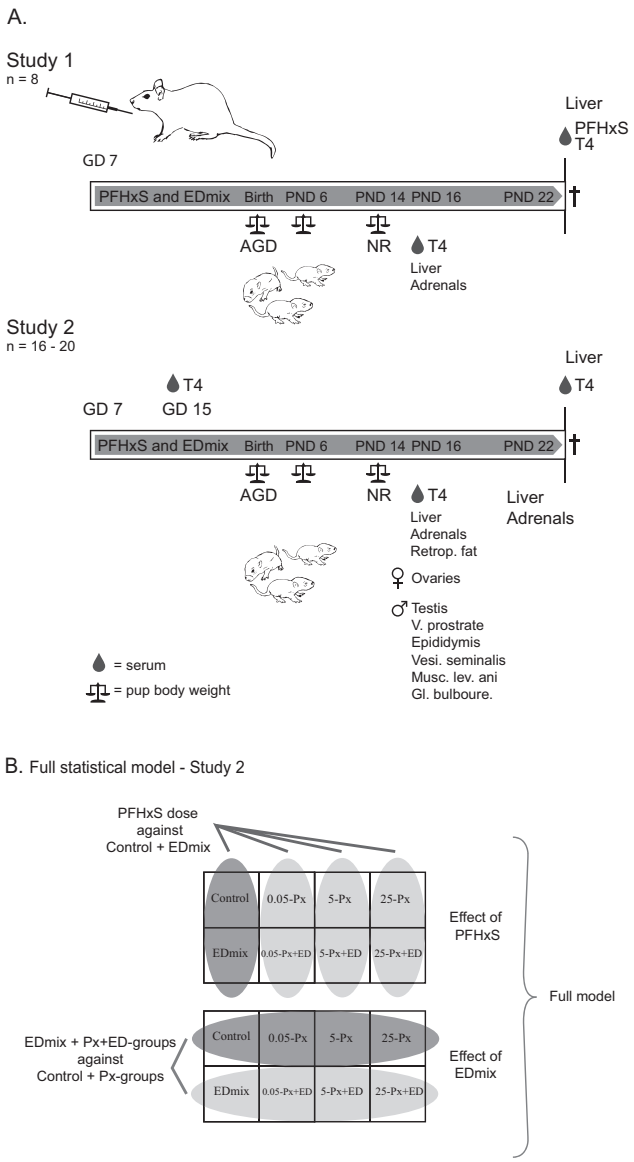

Figure 1. Study designs. A, Study design: Study 1 and 2. Two reproductive toxicity studies were performed as shown. The endpoints for dams and offspring are depicted along with timepoints. Exposure of dams to vehicle, perfluorohexane sulfonate (PFHxS), and/or a mixture of EDCs (EDmix) from gestational day 7 (GD) through postnatal day 22 (PND) except day of delivery. B, The full statistical model in Study 2 was modelled on the 2 × 4 design matrix depicted, this model allowed for comparisons of PFHxS dose against no PFHxS exposure (control and EDmix groups) and for EDmix exposed groups against no EDmix groups (control and PFHxS exposed groups). AGD, anogenital distance; NR, nipple retention; T4, thyroxine. *n* = time-mated dams.

Animals and Dosing

Study design of the two studies is presented graphically in Figure 1A. In both studies, time-mated nulliparous, young adult Wistar rats (HanTac: WH, Taconic Europe, Ejby, Denmark) were received on gestation day 3 (GD) of pregnancy (the day of plug-detection designated as GD 1) and randomly distributed for pairwise housing. On the day after arrival (GD 4) animals were pseudo-randomly allocated into groups with similar distributions in body weight. The expected day of delivery (GD 23) was termed PND 1 for the pups. Hence, the age of the pups was related to time of mating rather than day of birth, but the two timepoints were rather similar as only 5 out of 30 births in Study 1 and 13 out of 128 births in Study 2 did not occur on GD 23.

Table 2. Dosing

Group	PFHxS (mg/kg bw/day)	EDmix (mg/kg bw/day)	Time-Mated Dams	Viable Litters
Study 1				
Control	—	—	8	5
25-Px	25	—	8	6
25-Px+ED	25	32.11	8	5
45-Px	45	—	8	7
45-Px+ED	45	32.11	8	7
Study 2				
Control	—	—	20	20
EDmix	—	32.11	16	13
0.05-Px	0.05	—	20	16
0.05-Px+ED	0.05	32.11	16	13
5-Px	5	—	20	19
5-Px+ED	5	32.11	16	15
25-Px	25	—	20	17
25-Px+ED	25	32.11	16	15

Px, perfluorohexane sulfonate (PFHxS); ED, EDmix (mixture of EDCs, see Table 1).

Dosing of the dams was performed daily (between 8 and 10 AM) by oral gavage at a constant volume of 2 ml/kg bw/day from GD 7 through postnatal day 22 (PND), except the day of delivery.

In the range-finding study (Study 1), 5 groups of 8 animals were exposed to vehicle or 25 or 45 mg/kg bw/day PFHxS both with and without a background EDmix exposure (32.11 mg/kg bw/day) (Table 2). The main study (Study 2) consisted of 8 groups of 16–20 animals (Table 2) dosed with lower doses of PFHxS: 0.05, 5 and 25 mg/kg bw/day, both with and without a background EDmix exposure. Besides a vehicle control group, a group receiving only the EDmix was included in Study 2.

Study 1 was carried out in a single block, that is, dosing started for all animals at the same day, whereas Study 2 was subdivided into 4 blocks. Each block in Study 2 involved all treatment groups.

The animals were housed in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15 × 27 × 43 cm) with aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark), and Tapvei Arcade 17 (aspen wood) shelters (Brogaarden). The animals were kept pairwise from GD 3 to GD 17 and alone thereafter. From the day of arrival the housing standard controlled environmental conditions were: Reversed light/dark cycles of 12 h (light from 9 PM to 9 AM, dark from 9AM to 9 PM), humidity 55 ± 5%, temperature at 21 ± 1°C, and ventilation changing air 10 times per hour. All animals were fed ad libitum on a standard diet with Altromin 1314 (soy and alfalfa-free, Altromin GmbH, Lage, Germany) and were provided ad libitum acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast). The animals were inspected twice a day for overt toxicity. The body weights were recorded on GD 4 and daily during dosing from GD 7 onward to adjust the dose, to follow changes in weight gain, and to follow pregnancy status.

The animal experiments were carried out at the DTU National Food Institute facilities (Mørkhøj, Denmark). Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2015-15-0201-00553 C3. The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

AGD and Nipple Retention

On the morning after delivery the pups were counted, weighed, sexed, and checked for anomalies. Pups found dead were macroscopically checked for anomalies when possible. AGD was measured on all live pups with an ocular stereomicroscope. All offspring were counted and weighed again on PND 6 and PND 14. The number of nipples/areolas of the pups was counted on PND 14. The NR in male pups was defined as the number of areolas/nipples (a dark focal area with or without a nipple bud), present where nipples are usually located in female offspring. AGD and NR retention measurements were performed by the same experienced technician blinded to exposure group.

Autopsy

Offspring. Autopsy in Study 1 took place on PND 16 where up to 1 male and 1 female pup from each litter were sacrificed, trunk blood collected for plasma, and liver and adrenal glands excised and weighed. In Study 2, sacrifice was performed on PND 16 and PND 17 for male and female pups, respectively. If available, 1 pup per gender and litter was sacrificed for macroscopic examination, organ removal and blood collection. Liver, adrenal glands, and one retroperitoneal fat pad was excised and weighed from each pup, so was ovaries from female pups and sex organs from male pups (testis, ventral prostate, epididymis, vesicular seminalis, musculus levator ani, and glandula bulbourethralis). On PND 22, up to 1 male and female pup per litter was chosen for macroscopic examination and organ removal. Liver and adrenal glands were excised and weighed.

Dams. In both studies dams with viable litters were sacrificed at PND 22. Dams were weighed, anesthetized with CO_2/O_2, decapitated, and trunk blood collected for thyroid hormone analysis (see below). Uteri of the dams were excised and the number of implantation scars was registered. Livers were excised and weighed. All dams not giving birth were sacrificed, as described above, on PND 3 and implantation scars registered to exclude non-pregnant from the study and include dams with full-litter resorption in the determination of postimplantation loss and perinatal loss.

Thyroxine

In both studies trunk blood was collected from dams on PND 22 and from male and female pups on PND 16/17 in, respectively, 10 and 4 ml vacutainer tubes with sodium heparin. Additionally, on GD 15 in Study 2 tongue blood was drawn from the dams without anesthesia in heparinized Eppendorf tubes. All samples were stored on ice until centrifugation for 10 min at 4°C and 4000 rpm (rounds per minute). The plasma was collected and stored at −80°C until analysis for T4 levels by electrochemiluminescence-immunoassay (ECLIA)—photoncount at the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark using a Cobas 8000 E-modul.

All dams in Study 1, and 15 dams from each exposure group in Study 2 (20 controls) were sampled randomly for analysis. In Study 1 pup T4, PND 16, was determined based on litter means (from up to 1 male and 1 female pup per litter) and measured in control and PFHxS-only groups. In Study 2 each litter was represented by either a male or female pup (the different sampling times for male and female pups impedes conclusions on sex-specific effects) and was determined in the control, all PFHxS-only groups and the 25-Px + ED group.

Chemical Analysis

Internal PFHxS levels in the serum from dams were determined at the end of the dosing period (Study 1, sacrifice on PND 22), at Environmental Medicine, University of Southern Denmark (SDU), by online solid-phase extraction and analysis, using high-pressure liquid chromatography with tandem mass spectrometry. The analysis was performed by a slightly modified version of the method described by Haug et al. (2009) (Haug *et al.*, 2009). The serum samples were diluted 20–40.000 times prior to extraction to quantify within the calibration range of the method, normally used for human serum samples (Grandjean *et al.*, 2012).

Statistical Analysis

Data from continuous endpoints were checked for normal distribution and homogeneity of variance and then analyzed by analysis of variance (ANOVA). If assumptions were not fulfilled, data was transformed accordingly. Body weight and litter size was included as covariates in data analyses when considered as relevant (e.g., organ weights, AGD, and birthweight). If data from more than one pup per litter were available, litter was included as an independent, random, and nested factor in ANOVA or analysis was based on litter means.

The number of nipples/areolas (NR) was assumed to follow a binomial distribution and analyzed according to Hass et al. (2007) (Hass *et al.*, 2007). Statistical significance were assessed using multiple contrast tests (global error rate $\alpha = 5\%$, two-sided) (Bretz *et al.*, 2005). Here we used Dunnett contrasts to detect any dose-related effect and trend contrasts (linear, Helmert, reverse Helmert) assuming different dose-response shapes.

In Study 2, effect differences between the PFHxS-only and the control group and between PFHxS + ED and the EDmix group were tested by pairwise hypothesis testing, with the type I error controlled by the two-tailed Dunnett test (continuous endpoints) and Dunnett contrast (NR). Outcomes from this pairwise hypothesis testing were further investigated by integrating all exposures and control groups into one full linear model (2×4 design matrix, Figure 1B), with PFHxS and EDmix exposures including their interactions parameterized as indicator variables (i.e., magnitude of exposure is not considered). The advantage of the full model is not only a better control of false-positive decisions, but by assuming an identical data variability across all groups (which we consider fulfilled), it can result in more robust statistical outcomes. The chosen model parameterization allows explicitly making a statistical decision whether (and to which magnitude) EDmix changes in average the PFHxS responses across the different exposure groups. Furthermore, as a larger number of samples are considered in this full model, the increased sample size can identify much smaller effect changes than a simple pairwise comparison. The model was tested by a main factor for EDmix and dose-dependent interaction terms to account for non-parallelity between the PFHxS and PFHxS + EDmix dose-response pattern.

Outcomes from the full model analysis are only reported if they lead to different conclusions than the pairwise hypothesis testing.

SAS Enterprise Guide 4.3 (2010), SAS Institute Inc, Cary, NC, was used for statistical analyses.

RESULTS

Serum PFHxS

In Study 1, PFHxS was quantified in dam serum on PND 22 ($n = 5$–7, Figure 2). The average PFHxS concentration in the

control group was <0.1 μg/ml whereas 139 and 174 μg/ml PFHxS were measured in animals exposed at 25 and 45 mg/kg PFHxS, respectively. In the groups co-exposed to EDmix and PFHxS the PFHxS levels were slightly increased to 156 and 182 ug/ml, corresponding to a 12.7% and 4.9% increase, respectively (not statistically significant).

Pregnancy Data, Postnatal Growth, and General Toxicity

PFHxS and EDmix alone or in combination did not affect maternal weight gain during pregnancy, post-implantation loss, perinatal loss, litter size, and sex ratio (Tables 3 and Tables 4). In Study 2, the EDmix significantly increased postnatal maternal weight gain on PND 1–14 ($p = .0140$), whereas a reduced weight gain was indicated by Study 1. Male pup birth weight was slightly decreased by 25 mg/kg PFHxS in Study 2; in the full statistical model, exposure to a dose of 25 mg/kg decreased male pup birth weight by 3.5% ($p = .0351$) and this effect was also significant when comparing 25-Px + ED with the background EDmix group ($p = .0070$). In Study 1, birth weight was slightly reduced (6%, not statistically significant) in both the 25-Px + ED and 45-Px + ED groups compared with control (adjusted for litter size). There was no statistically significant effect on female pup birth weight in any of the studies.

Pup growth and weights on PND 6, 14, and 22 were unaffected in Study 1. In Study 2, slight significant decreases in body weight were seen in female offspring on day 6 and 14 at 5 mg/kg in the full statistical model ($p = .0209$ and $p = .0274$, respectively) and in the 5-Px + ED group compared with EDmix alone on day 6 ($p = .0121$). As decreased body weights were not seen at higher doses (25-Px + ED, 45-Px + ED), we evaluate these body weight changes as chance findings.

One pup from the EDmix group in Study 2 had a genital tubercle with features in between male and female and it had 9 nipples on PND 14. Upon sacrifice on PND 16 it had an underdeveloped testicle and an underdeveloped ovary. Having characteristics of an intersex pup (the first ever to be identified in our group), unrelated to exposure, it was excluded from the study.

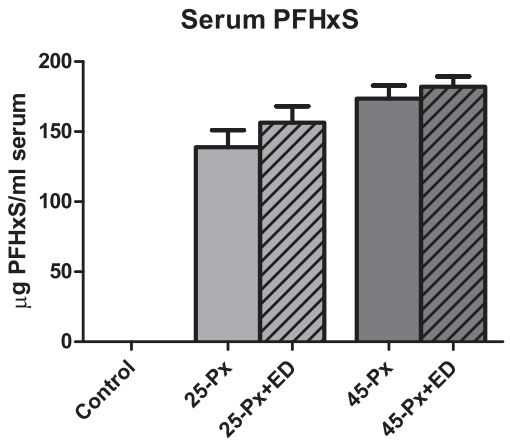

Figure 2. Dam serum PFHxS concentrations on PND 22 (Study 1). Control mean = 0.081 μg/ml, close to detection limit due to dilutions performed in the assay. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix. Data are shown as mean + SEM. $n = 5–7$.

Serum Thyroxine, T4

PFHxS clearly reduced total T4 levels in both dams and offspring. After only 7 days of gavage dosing (GD 15) the T4 levels in dams were significantly reduced to about 80% of controls at 5 mg/kg PFHxS ($p < .0001$), and to about 60% of controls at the highest dose (Figure 3A). These T4 reductions were similarly strong with the EDmix co-exposure. At later stages (PND 22), the T4 reductions were more pronounced, as they decreased to ca.70 and 30% of control levels at 5 and 25 mg/kg PFHxS, respectively ($p < .0001$). Furthermore, on PND 22 EDmix decreased significantly T4 levels by approximately 10% if data were analyzed

Table 3. Pregnancy and Litter Data: Study 1

	Control	25-Px	25-Px + ED	45-Px	45-Px-+ED
Time-mated females (no.)	8	8	8	8	8
Viable litters (no.)	5	6	5	7	7
Maternal bw GD 7 (g)[a]	231.1 ± 12.5	227.2 ± 12.9	225.4 ± 11.5	229.3 ± 9.5	227.8 ± 11.2
Maternal bw gain GD 7-GD 21 (g)[a]	80.7 ± 19.6	86.4 ± 18.0	85.2 ± 13.7	77.0 ± 8.4	71.0 ± 17.6
Maternal bw gain GD 7- PND 1 (g)[a]	15.5 ± 11.6	14.8 ± 6.1	19.0 ± 4.9	13.3 ± 10.9	15.6 ± 6.9
Maternal bw gain PND 1-PND 14 (g)[a]	40.8 ± 14.5	41.0 ± 17.0	34.4 ± 8.0	43.0 ± 8.9	33.9 ± 15.7
Gestational length (days)	23.2 ± 0.41	22.9 ± 0.45	22.8 ± 0.45	22.8 ± 0.39	23.0 ± 0.58
Litter size. Live pups PND 1 (no.)	10.0 ± 4.1	9.4 ± 4.6	10.8 ± 2.5	10.0 ± 2.3	7.9 ± 3.4
Postimplantation loss (%)	29.6 ± 40.1	13.9 ± 19.4	16.2 ± 6.7	26.9 ± 31.4	20.3 ± 17.1
Perinatal loss (%)	29.6 ± 40.1	26.9 ± 35.7	21.2 ± 11.1	27.8 ± 31.1	21.4 ± 16.9
Birth weight. Male pups (g)	6.7 ± 0.6	6.4 ± 0.5	6.3 ± 0.5	6.7 ± 0.5	6.6 ± 0.6
Birth weight. Female pups (g)	6.3 ± 0.6	6.2 ± 0.5	6.1 ± 0.6	6.0 ± 0.4	6.1 ± 0.5
AGD. Males (units[b])	22.1 ± 0.6	22.2 ± 1.4	20.9 ± 0.4	22.5 ± 0.9	23.0 ± 0.8
AGD. Females (units[b])	12.0 ± 0.9	12.1 ± 0.8	11.5 ± 0.5	11.7 ± 0.8	12.2 ± 1.0
Body weight. PND 6. Male pups (g)	14.0 ± 2.1	13.3 ± 1.1	13.2 ± 1.4	14.2 ± 2.4	13.8 ± 0.7
Body weight. PND 6. Female pups (g)	13.3 ± 2.3	12.9 ± 1.1	13.0 ± 1.3	13.1 ± 2.1	13.3 ± 1.2
Body weight. PND 14. Male pups (g)	31.7 ± 5.8	28.0 ± 2.1	29.6 ± 3.9	30.6 ± 6.3	30.3 ± 3.4
Body weight. PND 14. Female pups (g)	30.5 ± 6.2	27.6 ± 2.4	28.8 ± 3.9	29.2 ± 5.8	29.2 ± 4.2
Body weight. PND 22. Male pups (g)	53.9 ± 10.1	51.6 ± 6.9	52.0 ± 7.9	52.5 ± 10.3	51.5 ± 4.3
Body weight. PND 22. Female pups (g)	52.8 ± 9.5	50.2 ± 6.4	51.8 ± 7.3	50.4 ± 9.3	50.52 ± 3.9

Data represent group means based on dam or litter means ± SD. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix (mixture of EDCs, see Table 1); PND, postnatal day; GD, gestational day; AGD, anogenital distance.

[a]Only pregnant dams giving birth to viable litters.

[b]1 unit = 0.164 mm (i.e. male AGD of 22.1 units corresponds to 3.62 mm).

Table 4. Pregnancy and Litter Data: Study 2

	Control	EDmix	0.05-Px	0.05-Px + ED	5-Px	5-Px + ED	25-Px	25-Px + ED	Effect in Groups Exposed to EDmix Versus Groups Not Exposed to EDmix
Time-mated females (no.)	**20**	**16**	**20**	**16**	**20**	**16**	**20**	**16**	
Viable litters (no.)	**20**	**13**	**16**	**13**	**19**	**15**	**17**	**15**	
Maternal bw GD7 (g)[a]	227.2 ± 11.7	230.2 ± 10.8	225.0 ± 12.2	230.3 ± 9.9	227.4 ± 12.6	228.9 ± 12.0	231.9 ± 14.8	228.8 ± 11.9	
Maternal bw gain GD7-GD21 (g)[a]	84.0 ± 13.9	92.7 ± 15.7	87.0 ± 17.7	81.3 ± 17.0	85.1 ± 19.0	83.2 ± 22.1	87.9 ± 12.0	90.0 ± 9.5	
Maternal bw gain GD7- PD1 (g)[a]	21.0 ± 11.6	19.0 ± 8.5	23.3 ± 10.6	19.8 ± 11.3	19.9 ± 12.6	17.9 ± 12.0	17.8 ± 8.3	15.5 ± 7.8	
Maternal bw gain PND1-PND14[a]	34.3 ± 9.2	43.3 ± 8.9	35.5 ± 14.5	38.2 ± 16.1	35.3 ± 16.5	39.1 ± 11.4	37.7 ± 9.0	44.1 ± 10.9	↑
Gestational length (d)	23.0 ± 0.3	22.9 ± 0.3	22.9 ± 0.3	23.0 ± 0.3	22.9 ± 0.3	23.1 ± 0.4	22.9 ± 0.3	22.9 ± 0.4	
Litter size. Live pups. PD 1 (no.)	10.1 ± 3.6	11.8 ± 2.2	10.1 ± 3.8	9.5 ± 3.5	10.0 ± 3.5	10.0 ± 4.5	11.2 ± 2.1	12.2 ± 1.8	
Postimplantation loss (%)	5.9 ± 12.5	1.8 ± 3.4	5.8 ± 12.7	2.1 ± 4.1	4.3 ± 11.8	3.3 ± 7.6	7.0 ± 18.7	8.3 ± 8.4	
Perinatal loss (%)	13.3 ± 16.8	18.5 ± 24.5	23.4 ± 31.5	19.4 ± 25.8	9.6 ± 15.2	10.3 ± 18.4	14.6 ± 19.0	12.88 ± 11.84	
Birth weight. Male pups (g)	6.6 ± 0.5	6.6 ± 0.4	6.7 ± 0.5	6.7 ± 0.6	6.7 ± 0.5	6.6 ± 0.3	**6.5 ± 0.4**	**6.1 ± 0.5****	
							↓ compared with control + EDmix		
Birth weight. Female pups (g)	6.2 ± 0.5	6.2 ± 0.3	6.4 ± 0.5	6.4 ± 0.5	6.2 ± 0.5	6.1 ± 0.4	6.1 ± 0.4	6.0 ± 0.3	
AGD. Males (units[b])	22.7 ± 1.6	21.8 ± 1.2	23.0 ± 1.0	22.4 ± 1.3	22.6 ± 1.3	22.2 ± 1.0	22.1 ± 1.0	21.8 ± 0.9	↓
AGD. Females (units[b])	11.3 ± 0.6	11.2 ± 1.1	11.6 ± 0.7	11.2 ± 0.7	11.6 ± 0.9	11.8 ± 1.1	11.3 ± 0.5	11.3 ± 0.8	
Body weight. PND 6. Male pups (g)	13.7 ± 1.9	13.9 ± 1.4	14.1 ± 2.2	14.1 ± 2.2	14.2 ± 1.5	13.3 ± 1.5	13.4 ± 1.1	13.2 ± 1.1	
Body weight. PND 6. Female pups (g)	13.4 ± 1.5	13.3 ± 1.5	13.5 ± 1.9	13.6 ± 2.1	**13.4 ± 1.6**	**11.8 ± 1.9***	13.1 ± 1.2	12.9 ± 1.0	
					↓ compared with control + EDmix				
Body weight. PND 14. Male pups (g)	30.3 ± 5.3	29.7 ± 3.4	30.9 ± 5.3	31.1 ± 5.0	30.9 ± 4.4	28.9 ± 4.5	29.0 ± 3.8	28.5 ± 3.3	
Body weight. PND 14. Female pups (g)	30.2 ± 5.1	28.9 ± 3.3	30.4 ± 5.0	30.8 ± 4.7	**29.4 ± 4.3**	**26.6 ± 5.0**	28.5 ± 4.1	28.1 ± 3.3	
					↓ compared with control + EDmix				
Body weight. PND 22. Male pups (g)	51.5 ± 7.6	50.6 ± 5.5	52.8 ± 7.4	53.6 ± 7.7	52.3 ± 6.8	51.6 ± 8.8	50.5 ± 5.9	50.7 ± 5.9	
Body weight. PND 22. Female pups (g)	50.9 ± 7.5	49.4 ± 5.4	51.6 ± 7.3	52.3 ± 6.6	50.6 ± 6.0	46.9 ± 8.8	49.6 ± 5.8	49.4 ± 6.2	

Data represent group means based on dam or litter means ± SD. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix (mixture of EDCs, see Table 1); PND, postnatal day; GD, gestational day; AGD, anogenital distance.

[a]only pregnant dams giving birth to viable litters.

[b]1 unit = 0.1626 mm (i.e. male AGD of 22.7 units corresponds to 3.69 mm).

*$p < .05$ compared with EDmix; **$p < .01$ compared with EDmix; ↓ or ↑ $p < .05$ compared with indicated exposure.

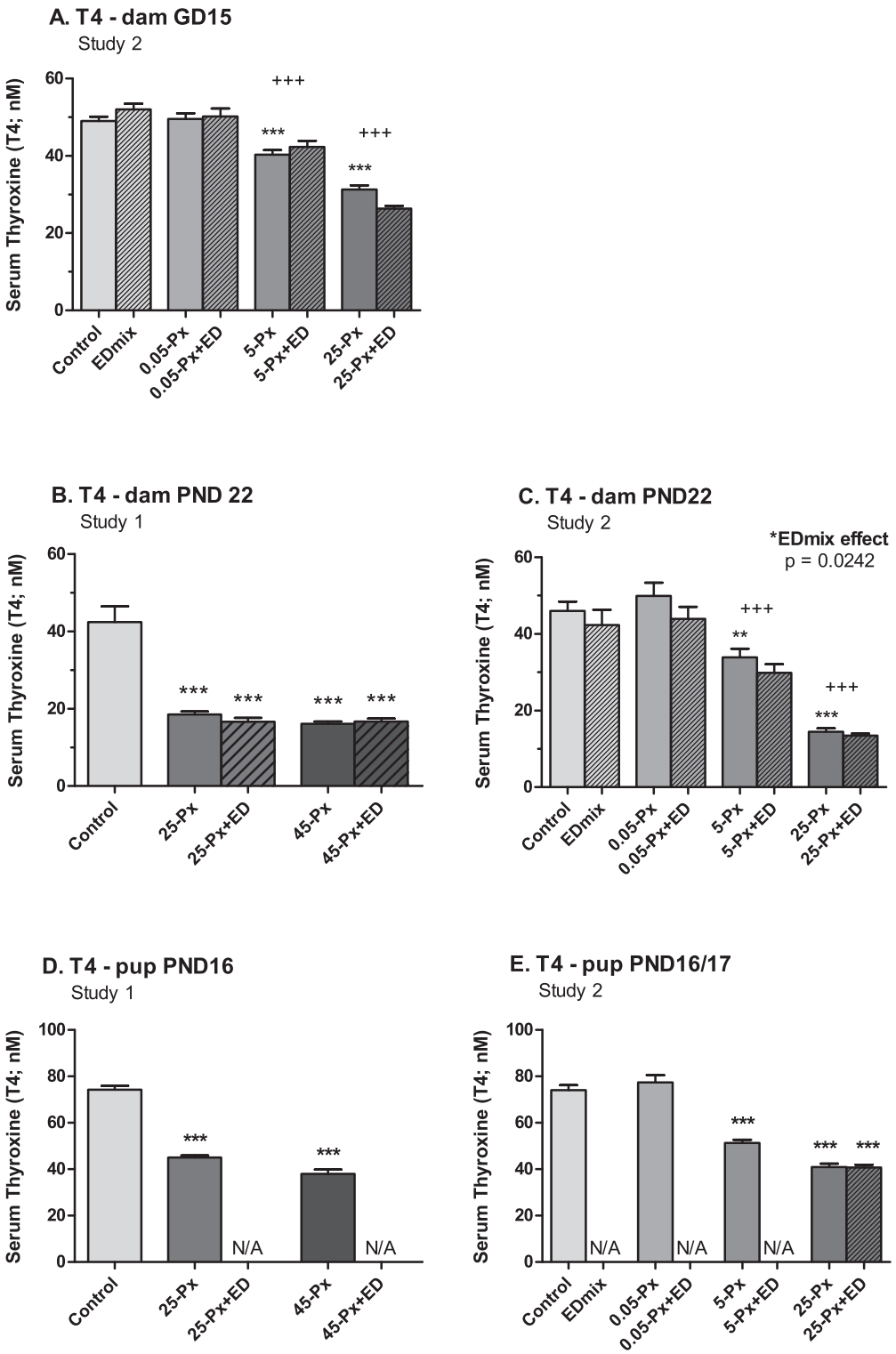

Figure 3. Serum thyroxine (T4) after exposure to PFHxS alone and in combination with a fixed dose of EDCs (EDmix). Dams were affected by both PFHxS and EDmix, pups only by PFHxS. A, Dam serum T4 on GD 15 was reduced from 5 mg/kg bw/day PFHxS. B, Dam serum T4 on PND 22 were reduced in all 4 exposed groups, Study 1. C, Dam serum T4 at PND 22, Study 2. The background EDmix exposure overall had a decreasing effect on T4 compared with no EDmix exposure ($p = .0242$). D, Pup serum T4 based on litter means of up to 1 male and 1 female pup per litter, Study 1. Doses of 25 and 45 mg/kg body weight reduced serum T4 on PND 16. E, Pup serum T4 on PND 16/17, Study 2. Doses of 5 and 25 mg PFHxS/kg body weight/day reduced serum T4 on PND 16/17. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix, N/A, not available. Data are shown as mean + SEM. A and C, $n = 13$–15 (except control with $n = 20$) for each group. B, $n = 5$–7. D, $n =$ litter means (from up to one male and one female pup per litter) of 5–7 litters. E, $n = 14$–18 litters represented by either a male or a female pup. ** $p < .01$ compared with control, *** $p < .001$ compared with control, +++ $p < .0001$ for full model comparison of indicated dose of PFHxS compared with no PFHxS exposure in the control and EDmix group. Comparisons of PFHxS+EDmix groups against EDmix not shown.

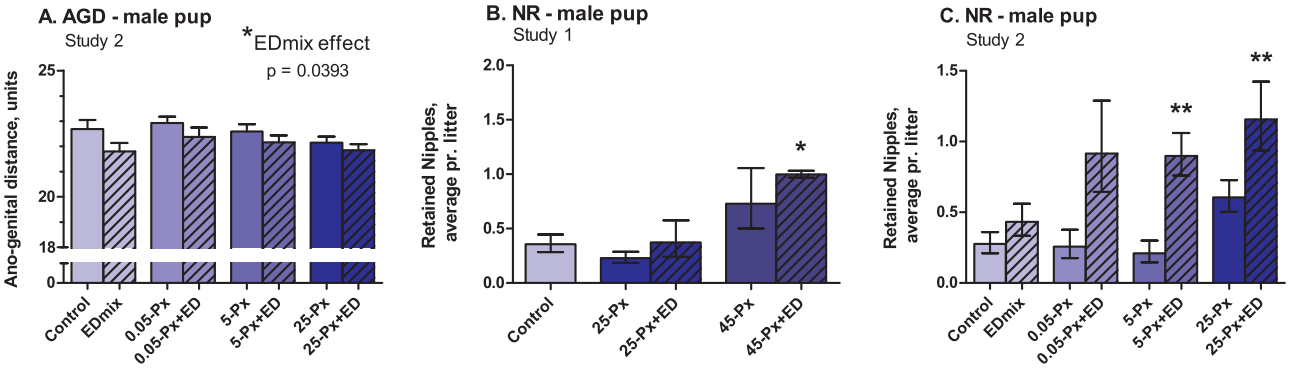

Figure 4. Anogenital distance (AGD, A) and nipple retention (NR, B–C) in male pups following gestational exposure to PFHxS alone and in combination with a fixed dose of EDCs (EDmix). EDmix significantly decreased AGD and NR was increased at high doses of PFHxS in the presence of the EDmix. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix. A, Data are shown as litter mean + SEM. B and C, Data are shown as litter mean ± SEM. A and C, n = 13–20 litters; B, n = 5–7 litters. AGD is expressed as units (1 unit = 0.1626 mm). Statistical analysis performed on data from all pups in each litter and adjusting for litter effects. * $p < .05$ compared with control, ** $p < .01$ compared with control.

in the full model ($p = .0242$) (Figure 3C). In Study 1, dam T4 levels were only measured on PND 22. Here the effects followed the same trend as in Study 2, however less pronounced, as the serum T4 levels were only reduced to app. 40% of the controls in all 4 exposure groups (Figure 3B).

T4 levels in the offspring were decreased after PFHxS exposure (Figs. 3D and 3E). In Study 1, pup serum T4 levels on PND 16 were decreased to app. 60 and 50% of controls in the 25 and 45 mg/kg PFHxS groups, respectively ($p < .0001$) (Figure 3D).

In Study 2, pup serum thyroxine was measured at PND16/17 in the control, the three PFHxS-only groups and in the high dose 25-Px + ED mix group. PFHxS significantly decreased serum T4 levels to app. 70% and 55% of control levels, in the 5 and 25 mg/kg PFHxS groups, respectively ($p < .001$) (Figure 3E). The EDmix had no additional decreasing effect on the T4 levels in the pups (all groups were compared with control only, a full model analysis was not possible as T4 was measured only in PFHxS-only groups and in the high dose 25-Px + ED mix group).

Anogenital Distance and Nipple Retention

PFHxS and EDmix caused weak effects on AGD and NR. In the smaller Study 1 no significant effects on AGD were observed (Table 3). In Study 2, AGD in male pups was slightly but statistically significant decreased by about 2% after exposure to EDmix ($p = .0393$, full model) (Figure 4A). No significant effects of PFHxS exposure was seen on male and female AGD.

For male pups from Study 1 the average number of nipples on PND 14 was significantly increased at highest PFHxS dose in combination with the background EDmix exposure (45-Px + ED, $p = .039$, Dunnett contrast) (Figure 4B).

In Study 2, PFHxS weakly, but significantly, increased NR at 25 mg/kg PFHxS when using trend analysis ($p = .01$ using Helmert contrast, and $p = .04$ using linear contrast) and in the full model ($p = .016$). NR was significantly increased when PFHxS exposure took place along with the background EDmix at the two highest doses (5-Px + ED and 25-Px + ED, $p < .01$, Dunnett contrast compared with control), and compared with the EDmix group ($p = .02$, linear contrast). When data from Study 2 were analyzed by the full model including all dose-dependent interaction terms, a high statistical significance of EDmix was revealed ($p < .01$, see Figure 1B for model visualization) indicating a strong overall effect of the EDmix on NR. NR in female pups was not significantly affected by exposure to either PFHxS or the EDmix.

Autopsy and Organ Weights PND 16 and PND 22

In both studies, no significant effects on dam or offspring body weights were seen at sacrifice on PND 16 or PND 22 (Supplementary Tables S1A and B).

Reproductive Organs. PFHxS had no effect on the weight of male reproductive organs examined on PND 16 in Study 2. EDmix exposure reduced the weight of the epididymides, ventral prostate and vesicula seminalis in the full statistical model ($p = .0023$, $p = .0055$, and $p = .0288$, respectively) (Figs. 5A–C). The remaining reproductive organ weights were not significantly affected by the EDmix (Figs. 5D–G). In females, no significant effects on ovary weights PND 17 or PND 22 were observed (Supplementary Table S1B). No reproductive organ weights were assessed in Study 1.

Retroperitoneal Fat Pad and Adrenals. Influences on weights of adrenals and the retroperitoneal fat pat were examined in the pups to evaluate potential influences on fat stores and steroidogenesis. In males, the weight of the retroperitoneal fat pad with body weight as a covariate was increased in male offspring exposed to PFHxS at 25 mg/kg ($p = .0309$, full statistical model) (Supplementary Table S1B). In a simple model the fat pad and the adrenal gland weight in the 25-Px group was also increased compared with control ($p = .0154$ and .0223, respectively). Overall, effects were not judged to be of major importance as the slightly lower body weights in the high dose group may have influenced the statistics, and no effects were seen in females, or in Study 1 males (Supplementary Table S1A), or at PND 22 (Supplementary Table S1B).

Liver. In both studies liver weights in offspring on PND 16/17 were increased at high doses of PFHxS (Figs. 6A and 6B and Supplementary Tables S1A and B). In Study 1, 25 and 45 mg/kg bw/day PFHxS caused higher liver weights in male on PND 16 compared with control, (Supplementary Table S1A). In males from Study 2 the effect of PFHxS was only significant when the full statistical model was applied or when PFHxS exposure took place along with EDmix exposure. This observation emphasizes the increased statistical power of the full model including all 8 groups and may indicate increased sensitivity to PFHxS when exposed to a background of chemicals (EDmix).

In Study 2 females, PFHxS increased liver weights in both the 5-Px and 25-Px groups compared with the control ($p = .0182$ and

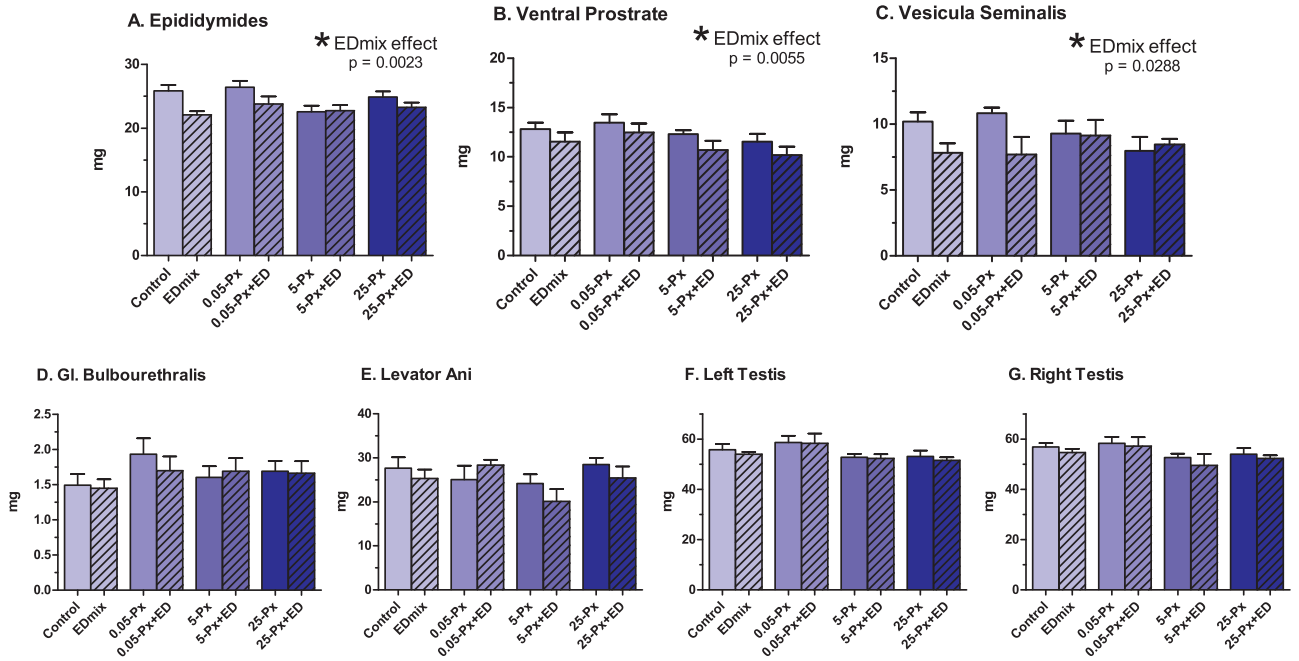

Figure 5. Male reproductive organs in male pups on PND 16 following gestational exposure to PFHxS alone and in combination with a fixed dose of EDCs (EDmix). PFHxS exposure had no effect on organ weights. The EDmix had an overall decreasing effect on the weights of the epididymides (A), ventral prostrate (B), and the vesicular seminalis (C), but no effects on glandula bulbourethralis (D), levator ani (E) or testis weight (F and G). Px, perfluorohexane sulfonate (PFHxS); ED, EDmix. Data are shown as mean + SEM. $n = 10–16$. * $p < .05$ compared with no EDmix exposure.

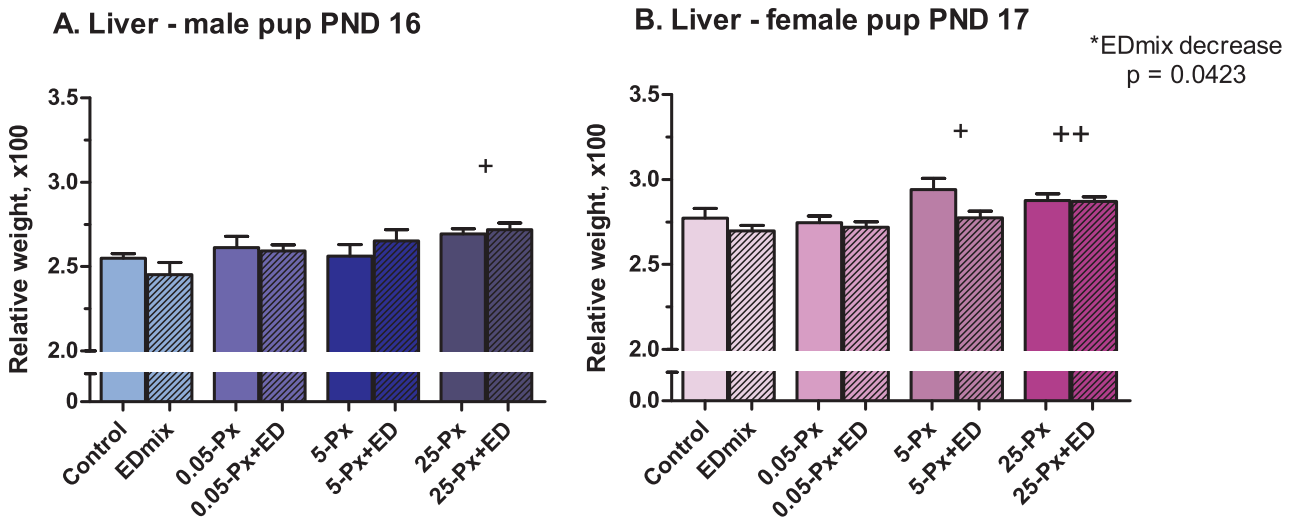

Figure 6. Pup relative liver weights after developmental exposure to PFHxS alone and in combination with a fixed dose of EDCs (EDmix). There was a linear relationship between liver weight and body weight male (A) PND 16: $R^2 = 0.85$ and female (B) PND 17: $R^2 = 0.91$). Therefore, to ease visual inspection, relative weights of the livers are depicted along with the results of the statistical analysis on relative weights, the absolute weights can be found in Supplementary Table S1A and B). Relative liver weights PND 16/17 were increased by PFHxS in the presence of the background EDmix exposure (not shown) and when analyzed in the full statistical model comparing PFHxS exposure at 3 levels against no PFHxS exposure in the control and EDmix groups. Px, perfluorohexane sulfonate (PFHxS); ED, EDmix. Data are shown as mean + SEM. $n = 11–16$ for each group. + <0.05 and ++ <0.01 for full model comparison of indicated dose of PFHxS compared with no PFHxS exposure in the control and EDmix group.

$p = .0478$, respectively, Supplementary Table S1B) and in the full model. At PND 22, similar, although less pronounced, increases in liver weights were found in both sexes (Supplementary Table S1B). Although the EDmix appeared to reduce liver weights in Study 2 using the full statistical model, this may be due to a chance finding of high liver weights in the 5-Px group ($p = .0279$, Supplementary Table S1B).

No exposure-related effects on dam liver weight were observed in any study apart from a reduction in Study 2 by the lowest dose of PFHxS (compared with no PFHxS, full model $p = .0329$) and in the .05-Px + ED group compared with EDmix only ($p = .0156$, Supplementary Table S1B). These effects were judged to be driven by the rather high mean liver weight in the EDmix-only group and do not appear to represent any toxicity of the chemicals.

DISCUSSION

In the present study we have demonstrated that PFHxS can cause endocrine disruption in the thyroid hormone system of Wistar rats. Additionally, our results have shown that co-exposure to PFHxS and a mixture of environmentally relevant EDCs can affect NR, AGD and some reproductive organ weights, even if PFHxS or the mixture of EDCs on their own, showed no or only weak effects on these endpoints.

Thyroid Hormone Disruption

The consequences of decreased thyroid hormone levels or altered action during human brain development can be serious, even in the case of subclinical T4 reductions (Berbel *et al.*, 2009; Morreale de Escobar *et al.*, 2000). Such alterations in neurodevelopment may result in adverse behavioral effects and low IQ, outcomes with large consequences for healthcare expenses and life-time income (Bellanger *et al.*, 2015; Grandjean and Landrigan, 2014). To our knowledge this is the first study that demonstrates significantly decreased total serum T4 levels in rat dams and their offspring after PFHxS administration. These significantly lower T4 levels were seen at 5 mg/kg bw/day, after only 7 days of exposure, indicating that PFHxS is an effective thyroid hormone disruptor in rats.

The thyroid effects observed here are consistent with studies showing decreased T4 levels in rats exposed to the structurally similar compound PFOS (Chang *et al.*, 2008; Yu *et al.*, 2009a,b). Furthermore, hypertrophy/hyperplasia of the thyroid follicular epithelium was seen in male rats after 42 days of PFHxS exposure (Butenhoff *et al.*, 2009), supporting our findings of decreased T4 levels in dams. Butenhoff *et al.* suggested microsomal liver enzyme induction to be the cause of the observed effects, as marked increase in liver weight was seen in their highest dosed animals (Butenhoff *et al.*, 2009). Such liver effects were not seen in our dams, and another likely mode of action for the observed T4 reductions could be binding of PFHxS to the thyroid hormone transport protein transthyretin (TTR), a common mode of action for perfluorinated compounds (Ren *et al.*, 2016; Weiss *et al.*, 2009).

Dam T4 reductions became more marked with time, with further decrease at weaning (PND 22), compared with GD 15. Data from Study 1 indicated that at higher doses, T4 reductions plateaued around 40% of control levels.

The observed postnatal T4 decreases in offspring, seen at PND 16/17, were likely due to lactational transfer of PFHxS. This is supported by data from Butenhoff *et al.* showing that at doses of 10 mg/kg, fetuses on GD 21 had serum concentrations 26% lower than dams, whereas pups at later stages (PND 22) had serum PFHxS concentrations 56% higher than dams at GD 21 (Butenhoff *et al.*, 2009). Additionally some studies on human PFHxS exposure find that breast milk is an important route of exposure (Kärrman *et al.*, 2007; Sundström *et al.*, 2011).

The mixture of 12 environmentally relevant EDCs (EDmix) contained some chemicals that on their own can affect the thyroid hormone system. However, the doses of the compounds included in the EDmix were all well below the reported No Observed Adverse Effect Levels (NOAELs) for T4 effects (Klammer *et al.*, 2007; Liu *et al.*, 2015; O'Connor *et al.*, 2002; Schneider *et al.*, 2011; Seidlová-Wuttke *et al.*, 2006, 2005; Yamada *et al.*, 2004). Yet, in PND 22 dams the EDmix caused a small, but statistically significant decrease in T4 levels, an effect which may have been caused by combination effects of the single chemicals in the EDmix.

Antiandrogenicity

PFHxS led to a slight but statistically significant positive trend in male NR, indicating some endocrine activity. However, PFHxS alone did not cause adverse effects on AGD or reproductive organ weights at any dose. Interestingly, co-exposure to PFHxS and EDmix significantly increased NR at 5 mg/kg PFHxS, and led to overall significant effects of EDmix on male AGD and weight of the epididymides, ventral prostrate, and seminal vesicle. Thus, co-exposure of PFHxS to the EDmix seemed to enhance the antiandrogenic action of the EDmix, possibly due to altered toxicokinetics of EDmix as discussed further below.

In rats, alterations in AGD, NR, and prepubertal reproductive organ weights are good markers of antiandrogenic action, and predictive of adverse effects on the male reproductive system later in life (Christiansen *et al.*, 2008; van den Driesche *et al.*, 2011), with male NR often being the most sensitive marker of antiandrogenic exposure during fetal sexual differentiation (Christiansen *et al.*, 2009; Laier *et al.*, 2006).

A mixture of EDCs similar to the EDmix in this study has previously been shown to adversely affect reproductive development. In our previous studies, the mixture also included paracetamol, which in itself seems to possess antiandrogenic properties (Axelstad *et al.*, 2014; Kristensen *et al.*, 2011). In those studies, the lowest tested mixture dose contained 32.11 mg/kg per day of environmental chemicals (corresponding to 100 times human high-end exposure) plus 80 mg/kg paracetamol (close to maximally recommended human therapeutic doses) given during two developmental windows sensitive to antiandrogens. This mixture significantly affected the female reproductive system at the lowest dose (Johansson *et al.*, 2016) whereas 2–4.5-fold higher doses affected male AGD, NR, and prepubertal reproductive organ weights (Axelstad *et al.*, 2014). In the present study the EDmix consisted of only the 32.11 mg/kg of environmental chemicals and although no statistical significance was detected on male NR, AGD, and reproductive organ weights if compared directly with the control, the full statistical model on the basis of all data suggested that these endpoints were weakly affected. In our typical experimental design the direct effect comparison allows for the statistical detection of an average AGD reduction of at least 5% (Christiansen *et al.*, 2008; Isling *et al.*, 2014), whereas the full statistical model specifically adapted to the experimental design in this study was able to detect an approximately 2% decrease in AGD as statistically significant.

General and Reproductive Toxicity

In the range-finding study, doses up to 45 mg/kg PFHxS did not lead to overt systemic toxicity in dams or offspring. However, in male pups doses of 25 and 45 mg/kg PFHxS increased liver weights and induced modest but significant decreases in birth weight. Additionally, we found female pup liver weight to be increased already at 5 mg/kg PFHxS on PND 17 and at 25 mg/kg on PND 22. This indicates a potential for PFHxS-induced developmental toxicity at doses even below the 10 mg/kg that was previously reported as a NOAEL for effects in dams or offspring (Butenhoff *et al.*, 2009). The lack of other systemic toxicities of PFHxS is in contrast to the effects of PFOS, which caused >95% postnatal pup dead at 5 mg/kg (Lau *et al.*, 2003), possibly by acting as a surfactant in the lungs of the newborn rats (Lau, 2012).

Gender Differences and Toxicokinetics

Interestingly, Butenhoff et al. (2009) observed adverse effects on liver and thyroid gland after repeated PFHxS administration (3 and 10 mg/kg) in the parental males, but not in dams, fetuses, or

pups. They also found strong gender differences in internal PFHxS levels, with serum levels in the males at least 4 times greater than the adult females in the 10 mg/kg group. In addition their results indicate a gender difference in distribution of PFHxS as the liver to serum ratio of PFHxS was greater than 2.5 in male rats and below 0.5 in females and offspring (Butenhoff *et al.*, 2009). In our study, external doses of 25 and 45 mg/kg resulted in serum PFHxS concentrations in pregnant dams similar to those in adult male rats after exposure to 10 mg/kg (Butenhoff *et al.*, 2009). Indeed, the half-life of PFHxS in female rats is lower than in males (Kim *et al.*, 2016; Sundström *et al.*, 2012) with consequently less internal exposure. The major elimination route for PFHxS is through the urine and for PFOA it has been demonstrated that this secretion can be strongly increased via organic anion transporter(s) in the renal tubules that appear in female rats around puberty (Kudo *et al.*, 2002). If also true for PFHxS we can expect similar kinetics for prepubertal males and females and that might explain why we see no clear gender differences in thyroid and liver effects at PND 16/17.

Based on the observation of more marked effects on T4, antiandrogenic endpoints and liver weight in the groups co-exposed to EDmix and PFHxS, we hypothesize that co-exposure could cause changes to absorption, distribution, and/or excretion properties of either PFHxS or the EDmix (note that PFHxS is not expected to be metabolized). This hypothesis can be examined indirectly via serum PFHxS determinations from Study 1, where PFHxS serum levels appeared higher in groups with concomitant exposure to the mixture. The reason for the nonsignificant increase in internal serum PFHxS levels when administered in combination with other EDCs is unknown to us and we can only speculate that the EDC co-exposure may have interacted directly with PFHxS-specific kinetic processes such that more PFHxS was absorbed and/or less PFHxS eliminated. Similarly, it is conceivable that the increased chemical load in the animals exposed to both PFHxS and the EDmix may have exacerbated the antiandrogenic effects of the EDmix by decreasing the elimination of the mixture.

CONCLUSIONS

We have demonstrated that PFHxS can induce marked reductions in circulating serum T4 in rats, which at critical developmental stages can lead to altered brain morphology and adverse behavior. Additionally, we have shown that a mixture of environmentally relevant EDCs can cause antiandrogenic effects at a lower dose than previously reported. Interestingly, PFHxS and the EDmix appear to exacerbate the effects of each other despite different modes of action. This illustrates that risk assessment may underestimate the toxicity of a chemical when mixture toxicity and background exposures are not taken into account.

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SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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Supplemental Material

Table S1A. Body and organ weights – Study 1

	Control	25-Px	25-Px+ED	45-Px	45-Px+ED
Autopsy PND 16					
Male, n	5	5	-	5	-
Body weight (g)	35.8±5.8	30.5±1.2	-	33.4±9.3	-
Liver (g)	0.90±0.15	**0.84±0.05****	-	**0.90±0.27***	-
Adrenal glands (mg)	9.3±2.5	7.3±1.1	-	8.0±1.8	-
Female, n	5	6	-	7	-
Body weight (g)	33.7±8.0	31.8±3.2	-	32.7±8.5	-
Liver, female (g)	0.86±0.20	0.88±0.12	-	0.92±0.22	-
Adrenal glands, female (g)	8.6±3.2	8.1±1.1	-	8.6±3.2	-
Autopsy PND 22, dam, n	5	6	5	7	7
Body weight, dam (g)	266.6±16.8	271.0±15.3	262.8±5.5	273.3±11.5	261.6±14.4
Liver, dam (g)	11.9±1.5	14.5±4.4	13.0±1.0	13.3±0.5	12.2±2.3

Absolute organ weights. Note that when relevant adjustment for body weight was included in the statistical analysis. Data are shown as mean ± SD.
Px = Perfluorohexane sulfonate (PFHxS)
ED = EDmix (mixture of endocrine disrupting chemicals, see Table 1)
PND = Postnatal day
* $p < 0.05$ compared to control
** $p < 0.01$ compared to control

10 Table S1B. Body and organ weights – Study 2

	Control	**EDmix**	**0.05-Px**	**0.05-Px+ED**	**5-Px**	**5-Px+ED**	**25-Px**	**25-Px+ED**	**Effect in groups exposed to EDmix versus groups not exposed to EDmix**
Autopsy PND 16, male, n	16	10	14	11	14	13	14	14	
Body weight (g)	33.7±4.8	31.6±2.0	34.1±5.6	34.1±6.8	32.4±3.5	32.3±5.6	30.5±4.9	31.4±3.2	
Liver (g)	0.86±0.12	0.76±0.08	0.87±0.15	0.88±0.18	0.83±0.11	0.86±0.18	**0.82±0.14**	**0.85±0.09 #**	
							↑↑ compared to control + EDmix		
Retroperitoneal fat (mg)	57.2±18.4	60.9±9.7	70.5±27.0	66.4±21.6	60.9±15.5	63.6±24.6	**62.0±23.0 ***	**62.3±12.0**	
							↑ compared to control + EDmix		
Adrenal glands (mg)	8.2±1.8	8.5±1.1	9.2±2.1	8.9±1.8	8.0±1.4	8.4±1.7	**8.4±1.6 ***	8.3±1.2	
Epididymides (mg)	25.9±3.6	22.1±1.8	26.4±3.9	23.8±3.9	22.6±3.5	22.8±3.1	24.9±3.2	23.3±2.8	↓↓
Ventral prostrate (mg)	12.8±2.6	11.6±3.0	12.5±4.7	12.5±3.0	12.3±1.5	9.9±4.3	11.6±2.9	10.2±4.3	↓↓
Vesicula seminalis (mg)	10.2±2.9	7.8±2.3	10.8±1.6	7.7±4.4	9.3±3.7	9.1±4.3	8.0±4.0	8.5±1.5	↓
Gl. Bulbourethralis (mg)	1.5±0.6	1.5±0.4	1.9±0.8	1.7±0.7	1.6±0.6	1.7±0.7	1.7±0.5	1.7±0.6	
Levator Ani (mg)	27.6±9.9	25.3±6.3	25.1±11.8	28.3±4.2	24.2±7.7	20.2±9.9	28.5±5.7	25.4±9.7	
Left testis (mg)	55.7±9.2	53.9±2.7	58.6±9.6	58.2±13.3	52.7±5.2	52.3±6.1	53.1±8.7	51.6±4.4	
Right testis (mg)	56.8±6.4	54.7±4.3	58.3±9.3	57.2±11.9	52.6±5.9	49.6±16.2	54.0±8.9	52.3±4.5	
Autopsy PND 17, female, n	16	13	11	13	16	12	16	15	
Body weight (g)	35.7±5.6	34.6±4.3	33.9±4.9	36.9±6.0	34.5±5.1	32.7±3.7	33.3±4.6	33.5±3.7	
Liver (g)	1.00±0.23	0.93±0.12	0.93±0.13	1.00±0.17	**1.01±0.14***	**0.91±0.12**	**0.96±0.17***	**0.96±0.12 ##**	↓
					↑ compared to control + EDmix		↑↑ compared to control + EDmix		
Retroperitoneal fat (mg)	45.2±17.1	48.2±13.5	45.5±15.2	54.2±16.5	50.7±22.5	44.8±14.1	43.7±13.6	47.9±16.8	
Adrenal glands (mg)	10.8±2.2	10.5±1.5	10.4±1.7	10.5±2.1	10.2±1.7	9.2±2.1	10.0±2.1	10.2±1.9	

Ovaries (mg)	6.7±1.5	7.2±1.9	6.6±1.4	6.8±1.6	7.3±1.1	6.4±0.8	7.3±2.1	7.0±1.4	
Autopsy PND 22	**Control**	**EDmix**	**0.05-Px**	**0.05-Px+ED**	**5-Px**	**5-Px+ED**	**25-Px**	**25-Px+ED**	**Effect in groups exposed to EDmix versus groups not exposed to EDmix**
Male, n	17	10	12	10	15	11	14	13	
Body weight (g)	51.0±6.3	48.8±4.0	50.7±8.0	53.1±7.0	50.3±5.4	49.2±5.6	48.6±6.5	50.8±5.6	
Liver (g)	1.73±0.27	1.63±0.12	1.76±0.29	1.81±0.25	1.76±0.21	1.73±0.18	**1.75±0.33 ****	**1.81±0.24 #** ↑↑ compared to control + EDmix	
Adrenal glands (mg)	15.0±3.2	14.4±2.3	14.1±2.9	14.0±2.1	14.2±2.7	13.9±2.2	13.8±2.3	13.7±1.7	
Female, n	17	13	11	12	16	11	15	15	
Body weight (g)	49.6±4.6	49.5±5.3	49.4±6.9	52.1±2.1	49.5±5.8	48.7±6.0	49.2±6.0	49.7±5.9	
Liver (g)	1.71±0.23	1.68±0.22	1.74±0.28	1.80±0.27	1.75±0.18	1.71±0.16	**1.75±0.24**	**1.79±0.21 #** ↑ compared to control + EDmix	
Adrenal glands (mg)	16.0±2.7	15.8±1.7	14.8±2.3	16.4±2.9	14.7±3.4	15.2±1.7	15.0±3.0	15.2±2.3	
Ovaries (mg)	18.7±3.7	18.2±3.7	20.1±2.4	17.9±5.2	18.6±1.8	17.8±3.4	18.2±3.7	18.8±2.6	
Dam, n	20	11	18	14	18	15	17	14	
Body weight (g)	274.0±15.1	278.6±13.0	281.3±14.5	278.7±13.9	274.7±13.8	278.4±15.0	282.7±14.6	283.1±13.0	
Liver (g)	12.28±1.55	13.32±1.69	12.26±1.46	11.95±1.57	12.37±1.77	12.51±1.63	13.20±1.23	13.59±1.01	

Absolute organ weights. Note that when relevant adjustment for body weight was included in the statistical analysis. Data are shown as mean ± SD.
* $p < 0.05$ compared to control
** $p < 0.01$ compared to control
# $p < 0.05$ compared to EDmix
$p < 0.01$ compared to EDmix
↓ or ↑ $p < 0.05$ compared to indicated exposure
↓↓ or ↑↑ $p < 0.01$ compared to indicated exposure

4 Manuscript II

Evaluation of Thyroid and Neurodevelopmental Effects in Rats after Perinatal Exposure to Perfluorohexane Sulfonate (PFHxS)

Ramhøj L, Hass U, Gilbert ME, Wood C, Usai D, Vinggaard AM, Mandrup K, Christiansen S, Svingen T, Axelstad M. *Manuscript*.

Evaluation of Thyroid and Neurodevelopmental Effects in Rats After Perinatal Exposure to Perfluorohexane Sulfonate (PFHxS)

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Running Title: Thyroid hormone disruption by PFHxS

Key words: thyroid, hypothyroxinemia, neurodevelopment, perfluorinated.

ABSTRACT

Introduction

Thyroid hormones (TH) are critical for mammalian brain development. In humans, low maternal serum thyroxine (T4) levels are associated with neurological deficiencies and cognitive impairment. Perfluorohexane sulfonate (PFHxS) is a widespread environmental contaminant found in human serum, tissues and milk. We have shown that PFHxS decrease serum thyroxine in pregnant rat dams and their offspring. Here, we further investigate effects on the thyroid system, neurodevelopment, and combination effects of PFHxS and a mixture of environmentally relevant endocrine disruptors.

Methods

Perfluorohexane sulfonate (PFHxS, 0, 0.05, 5 or 25 mg/kg/day with and without EDmix, a mixture of 12 endocrine disruptors e.g. phthalates, pesticides, UV-filters, Bisphenol A and butyl paraben) was administered (p.o.) to Wistar rat dams (n = 16-20/dose group) from gestation day (GD) 7 through postnatal day (PD) 22. Organs were removed on PD 16/17 and PD 22 and a subset weaned for assessment in activity boxes at three life-satges and the radial arm maze in adulthood.

Results/discussion

PFHxS not only decreased serum T4 levels in dams and offspring but in the high dose also reduced T3 to 84% of controls in both dams (PD 22) and pups (PD 16). The hypothalamic-pituitary-thyroid (HPT) axis was not activated based on lack of effect on serum TSH, thyroid gland histology, weight, and thyroid gene expression levels. Developmental hypothyroxinemia did not appear to increase physical activity levels in young and adult offspring. However, the expected sex difference was absent on PD 115 in low dose PFHxS (0.05 mg/kg) and at high doses in combination with EDmix (5 mg/kg + EDmix and 25 mg/kg + EDmix). Slight effects on offspring learning and memory did not appear correlated to decreased TH levels during development.

Conclusions

PFHxS decreased circulating levels of T3 and T4 in pregnant rat dams and their offspring

without apparent compensation by the HPT axis. The thyroid hormone disruption was not associated with detectable learning and memory deficits. Rather, findings suggest that PFHxS may disrupt sexual differentiation of the brain. Standard behavioral assays appear insensitive to adverse effects on brain development caused by thyroid hormone disruption. Hence, there is a need for development of sensitive assays to protect human thyroid function and brain development.

Introduction

Thyroid hormones (TH) are critical for normal brain development, as correct spatiotemporal action of THs ensures proper neurogenesis, migration, synaptogenesis and myelination (Bernal, 2015; Zoeller *et al.*, 2007). Hence, decreased thyroid hormone levels or altered TH action during development can affect human neurodevelopment, and it may result in adverse behavioral effects and low intelligence quotient (IQ) (Boas *et al.*, 2012; WHO/UNEP, 2013). Outcomes which have serious consequences for the affected individual and which on a societal level have large consequences for healthcare expenses and lifetime income (Bellanger *et al.*, 2015; Grandjean *et al.*, 2014). Thyroid hormone balance can be altered by exogenous substances as chemicals, drugs and insufficient intake of iodide or by certain diseases. Disorders like congenital hypothyroidism and severe iodine deficiency have shown that absent or major reduction in thyroid hormone levels cause detrimental effects as cretinism and severe mental retardation. However, even subclinical T4 reductions in the mother can have effects on neurodevelopment and IQ of the child (Berbel *et al.*, 2009; Ghassabian *et al.*, 2014; Haddow *et al.*, 1999; Henrichs *et al.*, 2010; Korevaar *et al.*, 2016; Morreale de Escobar *et al.*, 2000) yet it has proven difficult to establish a clear relationship between low-grade developmental thyroid hormone disruption and adverse effects in rodent models (Axelstad *et al.*, 2011b, 2011a, Gilbert *et al.*, 2013b, 2008, 2013a; Harry *et al.*, 2014; Kodavanti *et al.*, 2010). This relationship is crucial for risk assessment and regulation of chemicals based on their potential for thyroid hormone disruption, as it appears that environmental chemicals often cause more subtle changes to the thyroid system than anti-thyroid drugs as propylthiouracil (PTU) (Boas *et al.*, 2012; Gilbert *et al.*, 2012; WHO/UNEP, 2013).

Fetal development encompasses development of the thyroid hormone system but the hormones have functions before the fetal thyroid gland is functional (Howdeshell, 2002). Hence, the fetus is dependent on placental transfer of thyroid hormones from early in gestation. By late gestation the fetus has a functional gland producing about 83% of T4 found in its circulation with the rest being supplied by the dam (Morreale de Escobar *et al.*,

1990). This means that both the thyroid homeostasis of the dam and the fetus have an influence on the thyroid status of the pup, and consequently, on fetal brain development. Not only general thyroid status but specifically T4 levels are strictly necessary for brain development as maternal T4 is the primary source of hormone in the fetus and because only T4, and not T3, is transported into the fetal brain (Bárez-López *et al.*, 2017; Grijota-Martínez *et al.*, 2011; Landers *et al.*, 2017; Morreale de Escobar *et al.*, 1988, 1990). The HPT axis of the dam does not necessarily compensate adequately for low T4 to ensure optimal supply of T4 to the fetus and brain (Gilbert *et al.*, 2013a; Hassan *et al.*, 2017; Meerts *et al.*, 2002; O'Shaughnessy *et al.*; Sharlin *et al.*, 2010).
After birth the neonate still have the T4 from the mother that it was born with, but the thyroid gland and the HPT axis is also functioning and supplies the necessary hormone (Howdeshell, 2002; Morreale de Escobar *et al.*, 1990). Rodents are born at what corresponds to late 2nd trimester of the human gestation, hence some of rodent brain development take place during the postnatal period (Zoeller *et al.*, 2004). Therefore, some adverse effects of TH insufficiency in rodents are dependent upon the xenobiotic being transferred from the milk to the pups and to give effects also on the pup TH system (Axelstad *et al.*, 2013, 2011b; Crofton, 2000; Crofton *et al.*, 2005; O'Shaughnessy *et al.*, 2018).

Traditionally, we expect the feedback loops of the hypothalamic-pituitary-thyroid (HPT) axis to react with increased secretion of thyroid hormone stimulating hormone (TSH) in response to reduced serum T4 levels (Bansal *et al.*, 2014; Fujimoto *et al.*, 1999; Hood *et al.*, 1999a; McClain, 1995; O'Shaughnessy *et al.*; Shibutani *et al.*, 2009; Vansell *et al.*, 2004). However, in the case of several industrial chemicals, especially PCBs and PBDEs, it looks as if even high exposures for a prolonged time, which results in severe serum T4 reductions, do not cause the expected activation of the HPT-axis with a subsequent increase in TSH (Bansal *et al.*, 2014; Goldey *et al.*, 1995; Klaassen *et al.*, 2001; Kortenkamp *et al.*, 2017; Yu *et al.*, 2009).

Poly- and perfluoroalkyl substances (PFAS) is one class of environmental chemicals which is frequently reported to cause TH disruption (Lau et al., 2007). PFHxS is repeatedly shown to be in the top three of PFAS in terms of human exposure

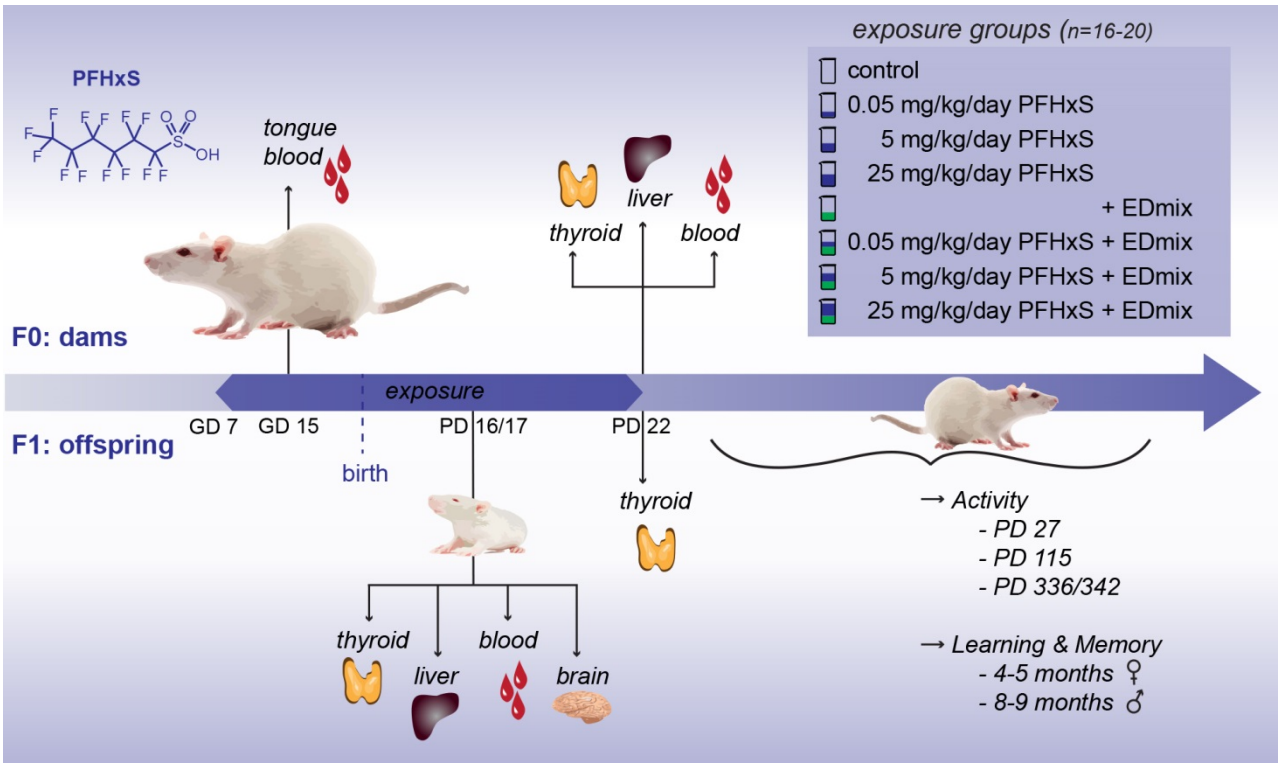

Fig. 1. Overview of the study. Time mated-rat dams were exposed to PFHxS and/or a mixture of environmentally relevant endocrine disrupting chemicals from gestation day 7 through pup day 22. Blood was taken from dams and pups for thyroid hormone assessments and organs were excised, weighed and stored for further analysis on PD 16 and 22. A subset of offspring was weaned (PD 22) and used for testing of motor activity levels at 3 ages and learning and memory in the radial arm maze (4-5-months of age for the females and 8-9 months for the males). The litter was used as statistical unit for all analyses. PFHxS: Perfluorohexane sulfonate, GD: Gestation day, PD: Pup day.

(Berg *et al.*, 2014; Glynn *et al.*, 2012; Kato *et al.*, 2011). Presently, the Mode of Action (MoA) by which PFHxS is thought to cause reductions in circulating TH levels is by binding to the serum transport protein transthyretin (TTR) (Ren *et al.*, 2016; Weiss *et al.*, 2009). This is also the case for other PFAS like PFOS and PFOA (Ren *et al.*, 2016; Weiss *et al.*, 2009), in addition other mechanisms may be relevant for PFHxS.

We recently reported (Ramhøj *et al.*, 2018) that developmental exposure to PFHxS caused marked reduction in total serum thyroxine (T4) levels in both dams and offspring. Here we report on additional thyroid hormone-related parameters, including T3 and TSH levels, thyroid gland weight, histopathology, targeted gene expression profiling, as well as neurodevelopmental consequences on behavior.

Materials and Methods

Animals and treatment

A developmental neurotoxicity study was performed with 144 time-mated wistar rat dams (See fig. 1). The study design has been described in details in Ramhøj et al., (2018, see Study 2). Briefly, the study consisted of 8 groups of 16-20 animals dosed with PFHxS (Perfluorohexane Sulfonate (PFHxS) purity >98 %, Sigma-Aldrich (Tridecafluorohexane-1-sulfonic acid potassium salt, CAS-No: 3871-99-6, lot #BCBC3545V). PFHxS doses were 0.05, 5 and 25 mg/kg bw/day, given both with and without a fixed dose of back-ground EDmix exposure as well as a control group and a group receiving only the EDmix. Corn oil (Sigma-Aldrich) was used as vehicle and dosing in the control group. The mixture of 12 endocrine disrupting chemicals (EDmix) consisted of 6 pesticides: Vinclozolin, Prochloraz, Procymidone, Linuron, Epoxyconazole, and Dichlorodiphenyldichloroeth-ylene, 2 UV-filters: 4-Methylbenzylidene camphor and Octyl methoxycinnamate, 3 plasticisers: Dibutyl phthalate, di-2-ethylhexyl phthalate and Bisphenol A, and 1 preserva-tive: Butyl paraben. The composition was based on concentrations found in highly exposed humans and it has been described previously (Christiansen *et al.*, 2012). However, acetaminophen (paracetamol) was omitted from the EDmix, which resulted in a total mixture dose of 32.11 mg/kg body weight (bw)/day (Ramhøj et al., submitted).
Animals were received on gestation day (GD) 3 of pregnancy and dosing of the dams was

performed daily by oral gavage from GD 7 to postnatal day 22 (PD), except the day of delivery. The expected day of delivery (GD 23) was termed PD 1 for all pups. Hence, the age of the pups was related to time of mating rather than day of birth.
From the day of arrival the housing standard controlled environmental conditions were: Reversed light/dark cycles of 12 hours (light from 9 pm-9 am), humidity 55 ± 5 %, temperature at 21 ± 1°C, and ventilation changing air ten times per hour. All animals were fed ad libitum on a standard diet with Altromin 1314 (soy and alfalfa-free, Altromin GmbH, Lage, Germany) and were provided ad libitum acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast).

Necropsy of up to one male and one female per sex per litter took place on PD 16 and PD 17, respectively, and again on PD 22 (see below). Also on PD 22 up to one male and one female pup from each litter were weaned and housed pairwise with an animal of the same sex and group (with a sibling if another animal of same sex and group were unavailable) and dams were sacrificed. This cohort of weaned offspring were subjected to behavioral assessment of motor activity levels at 3 ages and for the control and PFHxS-only groups learning and memory was tested in the radial arm maze. The study was terminated when the males were approximately 12 months and the females 13 months old.

The animal experiments were carried out at the DTU National Food Institute facilities (Mørkhøj, Denmark). Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2015-15-0201-00553 C3. The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Autopsies

Dams

On PD 22 dams were weighed, anesthetized with CO2/O2, decapitated, and trunk blood collected (see below). Livers were excised and saved for histopathological examination

(control and 25-Px). Thyroid glands were excised, weighed and saved for histopathology (control and PFHxS-only groups) in block 1-3. Thyroid glands from block 4 were stored in RNAlater at -80°C until extraction (see below) (control and PFHxS-only groups).

Offspring

On PD 16/17 and PD 22 pups were weighed, anesthetized with CO2/O2, decapitated, and trunk blood collected (see below). Livers from females were excised and saved for histopathology (PD 17). Male pup thyroid glands were excised with a piece of the thyroid cartilage for histopathology. Thyroid glands from females were weighed and from block 1 and 2 they were stored in RNAlater at -80°C until RNA extraction (see below) (control and PFHxS-only groups).

Thyroid hormones and thyroid stimulating hormone (TSH)

On GD 15, tongue blood was drawn from the dams without anesthesia. Trunk blood was collected from male pups on PD 16 and female pups on PD 17. Dam trunk blood was collected at weaning on PD 22. The blood was collected in heparinized Eppendorf tubes (GD 15) or in 4 or 10 ml vacutainer tubes with sodium heparin for the pup blood and the dam blood, respectively. Samples were stored on ice until centrifugation for 10 min. at 4°C and 4000 rpm (rounds per minute). The plasma was collected and stored at -80°C until analysis for T3 levels by electrochemiluminescence-immunoassay (ECLIA) – photoncount at the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark using a Cobas 8000 E-modul. 15 dams from each exposure group (20 controls) were sampled randomly for analysis. Each litter was represented by either a male or female pup (the different sampling times for male and female pups impedes conclusions on sex-specific effects).

Thyroid Stimulating Hormone (TSH) was measured in rat plasma with the Luminex RTHYMAG-30 (Merck Millipore) according to the instructions of the manufacturer on randomly chosen male (n = 11-13) and female pups (n =5-7) PD 16/17 (control, 5-Px and 25-Px groups only) and dams PD 22 (control and PFHxS-groups only).

Liver and thyroid gland histopathology

Liver

A predefined standardized slice of the liver was fixed in formalin, processed, embedded in paraffin, and 5µm sections were stained with haematoxylin and eosin. Livers from dams (on PD 22) and female offspring (PD 17) were evaluated for hepatocellular hypertrophy and vacuolation (macro- and microvesicular) in the control and high-dose (25 mg/kg PFHxS) groups.

Thyroid gland

Sections of thyroid glands from dams (PD 22) from control, Pax-25, EDmix, and Pax-25+EDmix, and male offspring (PD 16) from control group and Pax-25, were fixed in formalin, embedded in paraffin, and 5µm sections were stained with haematoxylin and eosin. Histology of thyroid glands were assessed for signs of pathological changes and scored accordingly as described below.

Thyroid glands from dams were assigned to one of the following four categories:

A: No remarks

B: Mild alterations with few irregular follicles or few follicles with multi-layered epithelium.

C: Follicular epithelial hypertrophy or hyperplasia, irregular follicles multi-layered follicular epithelium, papillary projections in follicular lumens and/or small peripheral follicles localized on the rim of the gland.

Thyroid glands from male pups were assigned to one of the following four categories:

A: No remarks – no changes

B: Mild alterations with few irregular follicles or few follicles with multilayered epithelium, increased cellularity.

C: hypertrophy or hyperplasia of follicular cells, irregular cells, multilayered follicular epithelium, papillary projections in follicular lumens

Thyroid gland and cortical gene expression

Both lobes of the thyroid gland from female PD 17 offspring were stored in RNAlater (Ambion AM7021) until RNA extraction. Thyroid glands from dams PD 22 were snap frozen in liquid nitrogen and stored at -80°C until analysis of one lobe. Upon sacrifice of male offspring on PD 16 an oblique slab of anterior to lateral cortex was stored in RNAlater at -80°C until RNA extraction. RNA was extracted with TRIreagent (Sigma T9424) according to manufacturer's protocol and glycogen added to facilitate precipitation of the thyroid gland RNA. RNA pellets were resuspended in nuclease-free water, DNased (RQ1 DNase, Promega M6101) and quantitated with Quant-iT RiboGreen RNA assay kit (Life Technologies R11490e). 2 µg RNA was reverse transcribed (Life Technologies High Capacity cDNA Reverse Transcription Kit 4374966) and the resulting cDNA was amplified using TaqMan Gene Expression Assays (Life Technologies) and TaqMan Gene Expression PCR Master Mix (Life Technologies 4369510) according to manufacturer's protocol. Amplification was performed on an ABI model 7900HT sequence detection system in duplicates. TagMan assays for thyroid glands were: *Slc5a5* (NIS) (Solute carrier family 5, Rn00583900_m1), *Nkx2.1* (TTF-1)(NK2 homeobox 1, Rn01512482_m1), *Tpo* (Thyroid peroxidase, Rn00571159_m1), *Tshr* (Thyroid stimulating hormone receptor, Rn00563612_m1), *Pax8* (Paired box 8, Rn00579743_m1), *Dio1* (iodothyronine deiodinase type 1, Rn00572183_m1) and for cortex samples: *Agt* (Angiotensinogen, Rn00593114_m1), *Col11a2* (Collagen type XI alpha 2 chain, Rn01428773_g1), *Gjb6* (Gap junction beta 6, Rn02042582_s1), *Hr* (Hairless, Rn00577605_m1), *Itih3* (Inter-Alpha-Trypsin Inhibitor Heavy Chain 3, Rn00569293_m1), *Klf9* (Kruppel like factor 9, Rn00589498_m1), *Ntf3* (Neurotrophin-3, Rn00579280_m1), *Pvalb* (parv) (Parvalbumin, Rn00574541_m1). *B2M* (Beta-2-microglobulin, Rn00560865_m1) and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase, Rn01775763_g1) were used as housekeeping genes

for thyroid gland and cortex, respectively. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Motor activity and habituation capability in offspring at 3 life stages

Motor activity was assessed in one male and one female pup from each litter on PD 27. The same animals were tested in young adult life on PD 115 and again in late life on PD 342/336.

The animals were placed individually in clean, empty cages with a flat rack lid and the cages were placed in activity boxes with photocells recording horizontal activity for 30 min. Neither food nor water was supplied during the testing. Movement was registered as interruption of a photobeam and data automatically collected by a computer in an adjoining room. The data collection started after 10 seconds habituation and continued for 10 periods of 3 minutes amounting to a total of 30 minutes observation. Data was collected as activity counts and break counts. Break counts being the total number of interrupted photobeams, and activity counts being disruption of adjacent photobeams, theoretically registering if the animal was moving around and not just continuously interrupting the same beam. The total activity during the 30 minutes was used as a measure of general activity. For habituation assessment data was divided into three periods: period 1 (1-9 min.), period 2 (10-21 min.), and period 3 (22-30 min.).

Radial arm maze

Offspring from the PFHxS-only groups: control, 0.05 mg PFHxS, 5 mg PFHxS and 25 mg PFHxS, were tested in the radial arm maze in adult life. One male and one female from each litter was tested, the females at 4-5 months of age and males at 8-9 months of age. Due to technical difficulties the radial arm maze was reduced to 7 arms. In the pre-testing week the animals were fed only 15 grams of chow a day in the afternoon and hand-fed a raw peanut (Brogaarden, Denmark) to accustom them to the taste. Following the pre-testing week the animals were tested once a day in the radial arm-maze, the animals were

tested until they had reached the end of all arms or until 10 mins had been spent in the maze. Testing was performed for 5 consecutive days, followed by a 2-day break for a total of three weeks. Time spent in the maze, errors (a visit to an arm already visited) and sequences (visits to adjacent arms) were registered and analysis performed based on animal totals and as means per day for week 1, 2 and 3.

Statistical Analysis

Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). Data were transformed if normal distribution and homogeneity of variance was not present. When relevant, body weight was included as a covariate in the analyses, e.g. when testing terminal organ weights. Histology results were analyzed with Fisher's test when analyzing 2 groups and Chi square tests for more than two groups (dam thyroid glands).

To avoid litter effects no more than one pup per litter was included in any analysis. Where an overall significant treatment effect was observed, Dunnet's test was used for two-tailed comparison with the alpha level for statistical significance always set to 5%. Exposure groups were tested against the vehicle control and in addition a "full model" was constructed; the study design with dose response exposure to PFHxS and the addition of groups exposed to both PFHxS and the EDmix allowed for construction of a statistical model integrating all exposures and control groups into one full linear model depicted and described in detail in Ramhøj et al., 2018, it includes PFHxS and EDmix exposures including their interactions parameterized as indicator variables. The chosen model parameterization allows explicitly making a statistical decision whether (and to which magnitude) EDmix changes in average the PFHxS responses across the different exposure groups. Furthermore, as a larger number of samples are considered in this full model, the increased sample size can identify much smaller effect changes than a simple pairwise comparison.

SAS Enterprise Guide 4.3 (2010), SAS Institute Inc, Cary, NC 27513, USA, was used for statistical analyses.

Results

Thyroid hormones in dams and offspring

We have previously reported dose-dependent reductions of dam and pup total serum T4 levels after developmental exposure to PFHxS (Ramhøj *et al.*, 2018), data are replicated here as % of control for reader discretion, Fig 2. Here we add T3 determinations to complement the characterization of the thyroid hormone system. On GD15 dam T3 levels were reduced to 97% at 25 mg/kg PFHxS using the full statistical model ($p = 0.0024$, data not shown). Whereas, on PD 22 dam T3 levels were further reduced to 84% of control levels in the 25 mg/kg PFHxS group. This reduction was statistically significant both using a simple statistical model and the full model ($p = 0.0099$ and $p = 0.0054$, respectively, fig 2 A).

As previously reported (Ramhøj *et al.*, 2018) pup T4 levels were down to 70 and 55% of control in the 5 and 25 mg/kg group, respectively. 0.05 mg/kg PFHxS nominally increased T4 levels to about 105% of controls, but this was not statistically significant. Opposed to the situation in the dams the EDmix had no effect on pup T4 levels. Here we report that T3 was dose-dependently affected in the pups with a reduction to 93% of control level at 5 mg/kg PFHxS (not statistically significant) and reductions to 84 and 87% of control levels in the 25-Px and 25-Px+ED groups, respectively ($p = 0.0002$ and $p = 0.0015$, respectively, Fig. 2 B). EDmix had no effect on pup T3 levels.

The hypothalamic-pituitary-thyroid axis

Offspring

TSH determinations on pups PD 16/17 showed no significant changes in any dose group compared to controls (Fig. 2 C, note the large control variability).

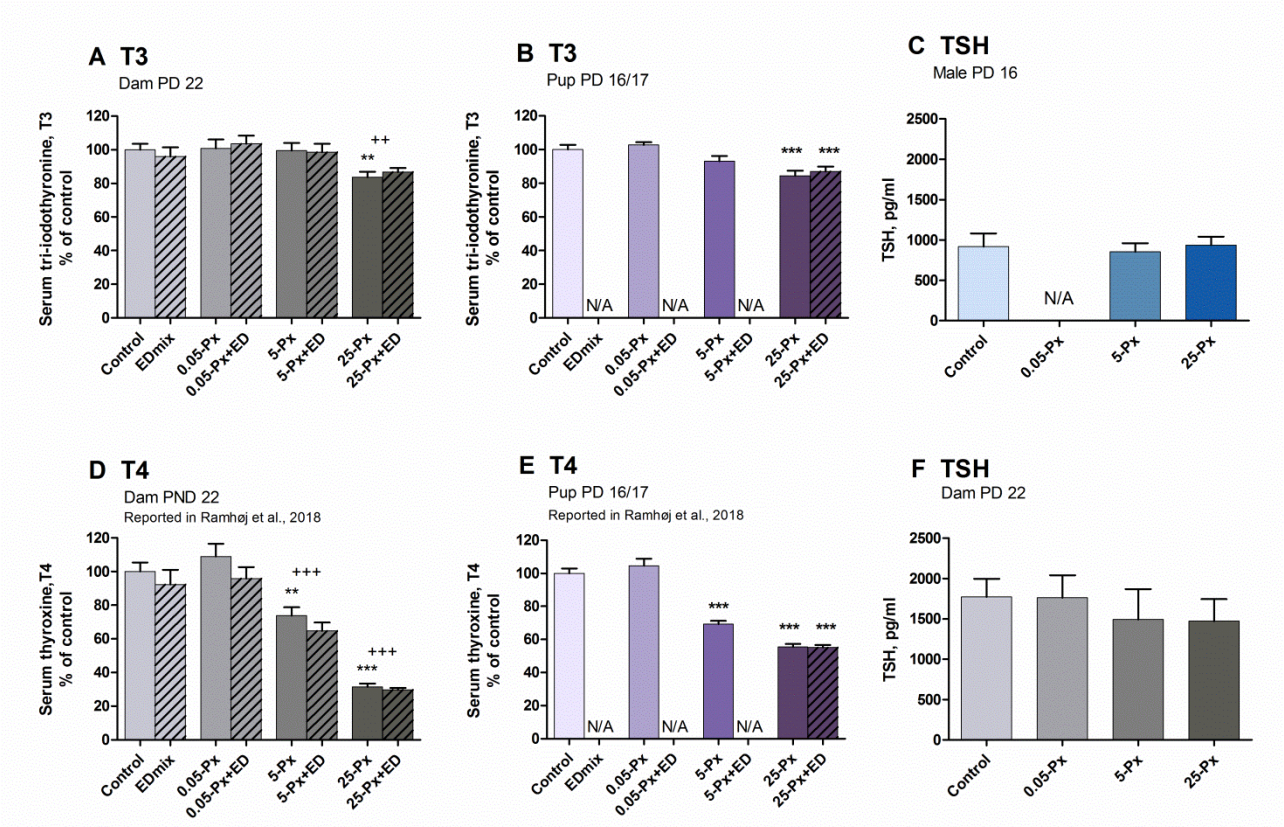

Fig. 2. T3 and TSH after developmental exposure to PFHxS. A. T3 levels in dams on PD 22 after exposure since GD 7. Data shown as mean + SEM. n = 13-15. B. PFHxS decreased Pup T3 levels at 25 mg/kg on PD 16/17. Data shown as mean + SEM. n = 14-18 litters represented by either a male or a female pup. C. TSH in male pups PD 16. Each symbol represents one animal with line and whiskers showing mean ± SEM. n = 11-13.
D. Dam serum T4 at PD 22 was reduced from 5 mg/kg PFHxS (absolute values previously reported in (Ramhøj *et al.*, 2018). n = 13-15 (except control with n = 20). E. Pup T4 was reduced form 5mg/kg PFHxS (absolute values previously reported in (Ramhøj *et al.*, 2018). n = 14-18 litters represented by either a male or a female pup. ** p <0.01 compared to control, *** p <0.00 compared to control, ++ p <0.01 for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and EDmix group. +++ p <0.001 for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and EDmix group. TSH: Thyroid stimulating hormone. T3: Tri-iodothyronine, N/A: not available.

PFHxS dose-dependently reduced female thyroid gland weights at weaning on PD 22 (Fig. 3 B), already from 0.05 mg/kg in the full model and from 5 mg/kg compared to control in a simple model. This effect was not statistically significant in the PD 17 glands (Fig. 3 A).

RT-qPCR on the PD 17 thyroid glands showed no effect on gene expression levels of *Slc5a5*(NIS), *Nkx2.1, Tpo,* and *Tshr* in the tested PFHxS-only groups (data not shown). Control and high dose (25-Px) male thyroid glands from PD 16 were evaluated histologically and smaller changes were found on PD 16 in the 25-Px group (Fig. 3 C-F) as 50% of the exposed animals received a B-score (reflecting one minor alteration that could be within the normal range) compared to 12.5% in the controls. By PD 22 this slight effect was no longer seen, possibly due to recovery. No animals were given the C-score at any time (reflecting hypertrophy and/or hyperplasia).

We previously reported (Ramhøj *et al.*, 2018) increased liver weight in both male and female offspring exposed to PFHxS and/or EDmix during development (from 5 mg/kg PFHxS in female offspring). Here we followed up on these observations and histological assessment of liver sections from female offspring showed no differences between controls and exposed animals (25 mg/kg PFHxS). Minimal midzonal microvesicular vacuolation was found in female offspring, but the change was evenly distributed between exposed and control animals. Morphological liver changes that could explain the changes in liver weight were not found.

Dams

TSH in dams on PD 22 was not altered by exposure to PFHxS (Fig. 2 F).

There was no statistically significant effects on dam thyroid gland weights apart from a reduction by the EDmix ($p = 0.0133$, full model, data not shown). There was no changes to histopathology in the glands (data not shown) and gene expression levels of 6 genes involved in thyroid hormone economy (*Slc5a5*(NIS), *Nkx2.1, Tpo, Tshr, Pax8* and *Dio1*, data not shown) showed no indications for an increase in thyroid gland activation to compensate for the reductions in serum thyroid hormones.
Histological assessment of dam livers PD 22 showed no difference between control and high dose animals (25 mg/kg PFHxS).

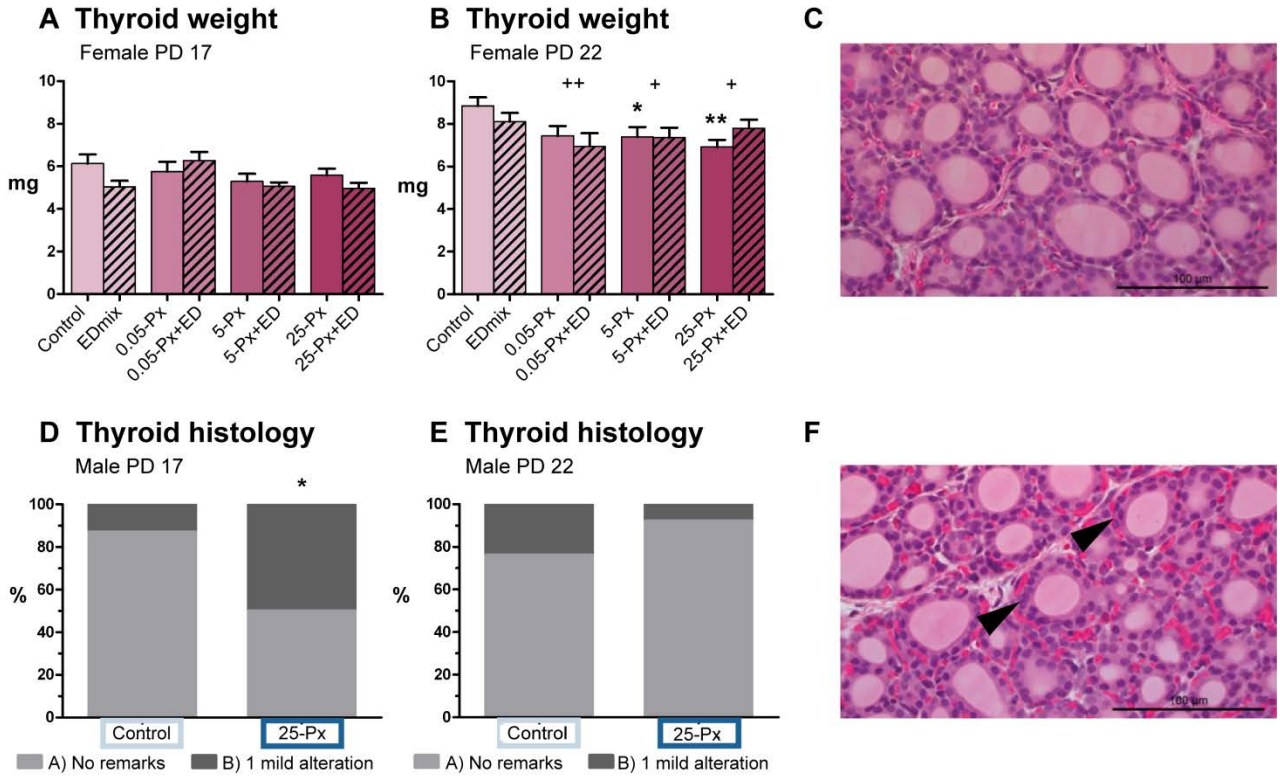

Fig. 3. Thyroid gland weight and histology PD 16/17 and 22, and histology on pup lives PD 16/17 after developmental exposure to PFHxS. A. and B. Female pup thyroid gland weight at PD 16 and PD 22. Data shown as mean + SEM. * p <0.05 compared to control, ** p <0.01 compared to control. n = 11-16.

D. and E. thyroid histopathology on male pup thyroid glands PD 16 and PD 22. Bars represents animals receiving indicated score. * p <0.05. n = 13-17 animals per exposure group at each time. C. and F. representative images of thyroid tissue from a control male pup (C) receiving a score of A (no remarks) and a male pup from the 25-Px group (F.) receiving a score of B (1 mild alteration, potentially within natural variation) for increased cellularity (marked with arrowheads).

Brain development

Cortical gene expression

The cortical expression of 8 thyroid hormone responsive genes previously shown affected after developmental PTU exposure was assessed in male PD 16 pups: *Agt, Col11a2, GJb6, Hr, Itih3 Klf9, Ntf3,* and *Pvalb* (Fig. 4 A) (O'Shaughnessy *et al.*). No clear evidence for decreased expression due to lack of thyroid hormone were observed. Expression levels of *Itih3* and *Klf9* were significantly increased and decreased, respectively, however, the modest effect sizes of less than 0.25-fold warrant further studies of these potential effects on the developing brain.

Motor activity

Effects on motor activity levels in all of the three tested ages were sparse and did not correlate with thyroid hormone levels during development. The most notable changes at the 3 ages are described below.

In prepuberty no sexual dimorphism was observed and no significant effects of PFHxS exposure were seen in either male of female offspring (Fig. 4 B).

In young adulthood the most notable changes were reduced activity in the middle test period in 0.05 mg/kg females ($p = 0.014$, compared to control)(data not shown). In males increased initial activity was seen in the 5 mg/kg group ($p = 0.018$, compared to control)(data not shown). The EDmix appeared to increase activity levels, in young adult male offspring total activity ($p = 0.0446$, full model, Fig. 4 C) and activity in the last half of the testing period ($p = 0.0297$, full model) were increased (data not shown).
Also in young adulthood the inherent sex-difference in activity had disappeared in the group exposed to low-dose PFHxS (both compared to control and in the full model) and in the higher exposure 5-Px+ED and 25-Px+ED groups (Fig. 4 C). Both indicating disrupted

sexual differentiation of the brain and observed at a very low dose of PFHxS or at higher doses in combination with the background mixture exposure.

In old animals adding the EDmix to the PFHxS exposure caused some small but significant changes (data not shown). 25-Px+ED reduced activity compared to EDmix in the old female offspring.
Whereas, again the EDmix appeared to increase activity, this time in the old female offspring where the EDmix group were more active than controls in the initial phase of the testing ($p = 0.0082$, t-test versus control) (data not shown).

Learning and memory in the radial arm maze

No evidence of a thyroid hormone mediated impaired learning was seen in the adult offspring. The only statistically significant effects observed were at 0.05 mg/kg PFHxS, where the males made fewer errors during the first week of testing (Fig. 4 E) and the females both during the first week of testing and overall spent more time in the maze (Fig. 4 D), both compared to their respective control. Conclusions on sex differentiation for learning and memory cannot be made due to the different ages at which females and males were tested.

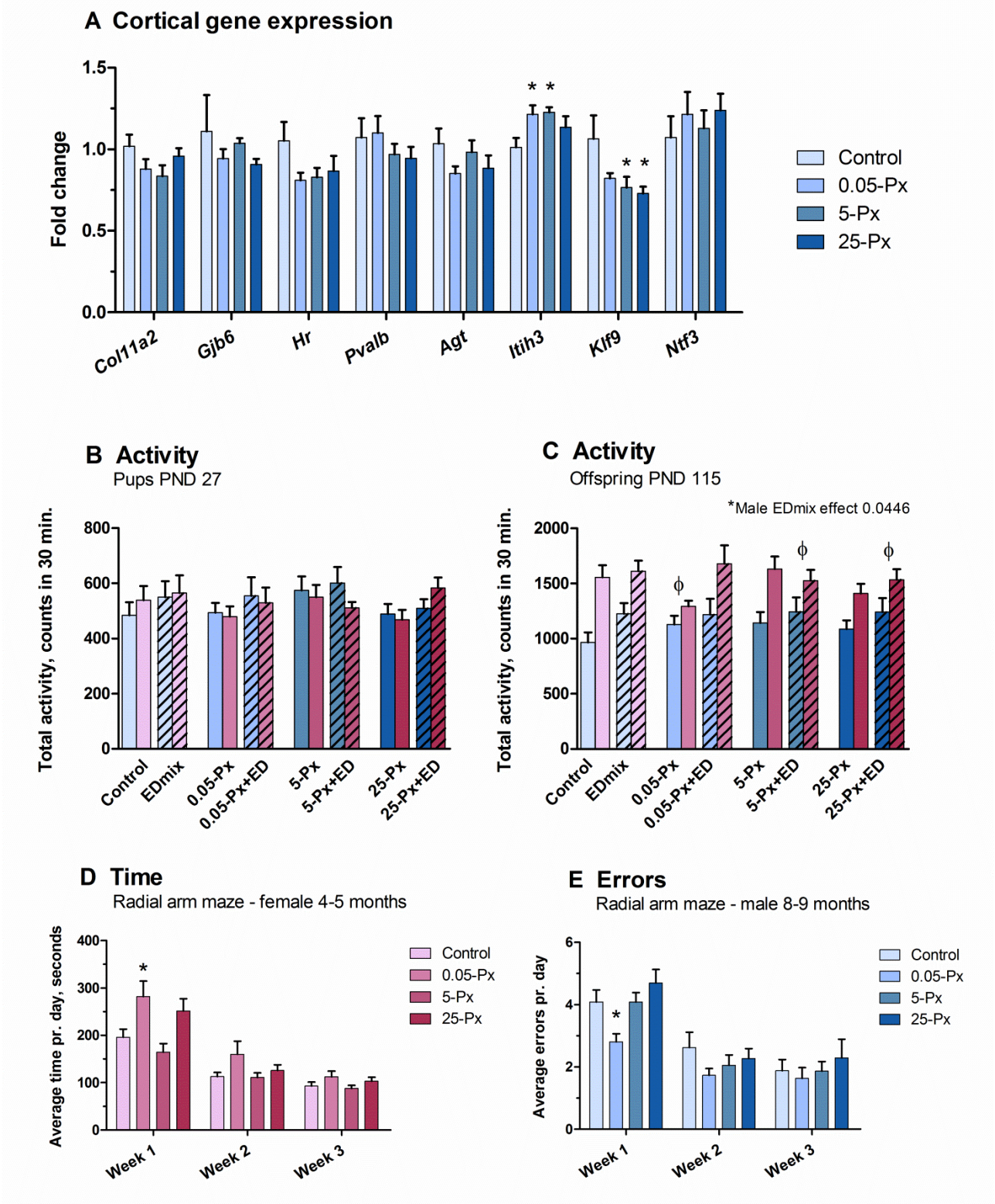
A Cortical gene expression
Fold change
1.5
1.0
0.5
0.0
* *
* *
Col11a2
Gjb6
Hr
Pvalb
Agt
Itih3
Klf9
Ntf3
Control
0.05-Px
5-Px
25-Px
B Activity
Pups PND 27
Total activity, counts in 30 min.
800
600
400
200
0
Control
EDmix
0.05-Px
0.05-Px+ED
5-Px
5-Px+ED
25-Px
25-Px+ED
C Activity
Offspring PND 115
*Male EDmix effect 0.0446
Total activity, counts in 30 min.
2000
1500
1000
500
0
ϕ
ϕ
ϕ
Control
EDmix
0.05-Px
0.05-Px+ED
5-Px
5-Px+ED
25-Px
25-Px+ED
D Time
Radial arm maze - female 4-5 months
Average time pr. day, seconds
400
300
200
100
0
*
Week 1
Week 2
Week 3
Control
0.05-Px
5-Px
25-Px
E Errors
Radial arm maze - male 8-9 months
Average errors pr. day
6
4
2
0
*
Week 1
Week 2
Week 3
Control
0.05-Px
5-Px
25-Px

Fig. 4 Effects on cortical gene expression, motor activity levels, and learning and memory after developmental exposure to PFHxS.
A. Cortical gene expression levels for thyroid hormone mediated genes in male offspring PD 16. n = 8-9.
B. and C. Motor activity levels in offspring PD 27 and PD 115. As expected there was an inherent sex difference in activity levels between control males and females in the young adult offspring, this sex difference was not found in the 0.05-Px, 5-Px+ED and 25-Px+ED groups, indicating disturbed sex differentiation of the brain. n = 11-19 animals per sex per litter. D. and E. Learning and memory assessed in the radial arm maze. n = 16-17 for the females, n = 15-19 for the males. Data shown as mean + SEM, * $p < 0.05$ compared to control, ϕ $p > 0.05$ for a sex difference within the indicated exposure group

Discussion

Reduction in TH and Mode of Action

PFHxS exposure caused marked reductions in both T4 (reported previously) and T3. The effects were seen in both dams and offspring, and were significant already after 7 days of exposure, however longer exposure caused even more pronounced effects (i.e. larger reductions were seen on PD 22 than on GD 15). The most severe effect was the T4 reduction in dams on day 22, which in study 2 was down to 30% of control levels (at 25 mg/kg bw/day). In study 1, where a smaller group size was used, exposure to both 25 and 45 mg/kg (Ramhøj *et al.*, 2018) caused T4 reductions down to approximately 40% of control levels.

The mode of action by which PFHxs causes such severe T4 reductions has not yet been investigated in depth, but PFHxS binds to both TTR and albumin (Kerstner-Wood *et al.*, 2003; Ren *et al.*, 2016; Weiss *et al.*, 2009). Therefore, displacement of thyroxine from serum transport proteins is expected to be the primary MoA for the PFHxS-induced hypothyroxinemia. This MoA has been further explored in rats for the similar perfluorinated compound PFOS, in a study where Chang et al., (2008) showed that transiently in the hours following administration of PFOS, free T4 rose dramatically, presumably due to displacement from transport proteins, followed by increased turnover and loss of thyroid

hormone through urine and feces (Chang *et al.*, 2008). Total T4 remained low during the remaining 7 days of the study and it was not accompanied by HPT-activation. These observations are in line with our findings, and it can be hypothesized that a reached steady state between serum protein bound T4 and PFHxS could account for the observed maximum reductions in serum T4 to 30% of control levels.

Another possible MoA is induction of the thyroid hormone glucuronidating enzyme UGT1A1 in the liver which in concert with increased thyroidal Dio1 mRNA, which the authors deemed responsible for the thyroid hormone effects after 91 days of PFOS exposure (Yu *et al.*, 2009). However, the effects on thyroid and liver were only evident from 5 mg PFOS/L drinking water whereas T4 were down to 60% of control already at 1.7 mg/L (Yu *et al.*, 2009). Our studies could not confirm increased Dio1 mRNA levels in the dam thyroids on PD 22. Additionally, PPARα, CAR and PXR pathways were activated in a PFHxS mice study (Chang *et al.*, 2018) and in a PFOS rat study (Dong *et al.*, 2016). PFHxS did not appear to bind TR in an *in vitro* study (Ren *et al.*, 2015), whether other molecular initiating events as inhibition of TPO or NIS or effects on peripheral deiodinases could be contributing to the TH effects, has to our knowledge not yet been investigated.

PFHxS reduced thyroid hormone levels in rats without activation of the HPT-axis

In spite of the very marked decreases in circulating T4 levels, no significant effects on TSH levels were measured in dams (PD 22) or in offspring (PD 16). Based on our present understanding of thyroid physiology and the HPT-axis, reduced circulating T4 levels are expected to cause increases in TSH signaling. This leads to increased thyroid gland activation, with consequent changes in thyroid gland gene expression, hypertrophy/hyperplasia of the thyroid follicular cells, and increases in thyroid gland weight (Hood *et al.*, 1999b; McClain, 1989; Vickers *et al.*, 2012; Zoeller *et al.*, 2007). The anti-thyroid drug PTU is a compound acting in this way, causing serum T4 levels to be reduced first, but soon followed by HPT axis activation. However, thyroid hormone disruptions by

environmental chemicals does not necessarily lead to an activation of the HPT axis (Kortenkamp *et al.*, 2017).

Here, the only effect in the thyroid system apart from reduced T4 and T3 were indications of adverse effects on male thyroid histopathology at PD 16. These were, however, transient (no longer seen on PD 22) and very mild, indicating that they could be chance findings within natural physiologic variation.

It is presently unknown whether prolonged or increased exposure to PFHxS would have caused an activation of the HPT-axis. It seemed to be the case in male rats exposed to 3 and 10 mg/kg PFHxS for 42 days as adverse effects on male rat thyroid gland histopathology was found. This indicates a potential activation of the HPT axis at higher exposures. Unfortunately, TSH levels or thyroid gland weights were not obtained (Butenhoff *et al.*, 2009). The effects on male histopathology, compared to females, can be explained by a high internal exposure with high serum PFHxS levels, clear signs of toxicity and a different organ distribution of PFHxS, due to a poor renal elimination in males (Kim *et al.*, 2016; Sundström *et al.*, 2012). In mice, at somewhat lower PFHxS internal exposures, there was no effect on serum TSH, thyroid gland weights or histopathology, but T3 and T4 were not measured (Chang *et al.*, 2018). Long term exposure to PFOS did not result in significantly increased TSH levels (Yu *et al.*, 2009).

It is thus clear that while we may be unsure of the long-term and high dose effects of PFHxS we know that it does not activate the HPT axis in a manner similar to PTU. It seems possible that the PFAS, like certain PCBs and PBDEs, can markedly reduce circulating T4 levels without a compensatory response within the HPT axis, even after long term exposure (Bansal *et al.*, 2014; Goldey *et al.*, 1995; Klaassen *et al.*, 2001; Kortenkamp *et al.*, 2017; Yu *et al.*, 2009).

PFHxS induced hypothyroxinemia and effects on the developing brain

There was no correlation between circulating T4 levels during development and behavioral effects measured in prepuberty or adulthood. While for PTU, MMI or thyroidectomy there is a correlation between developmental T4 levels and a wide range of behavioral effects in rats (Akaike *et al.*, 1991; Axelstad *et al.*, 2008; Crofton, 2004; Gilbert *et al.*, 2006; Goldey *et al.*, 1995; Kobayashi *et al.*, 2005). On the other hand, despite validated developmental neurotoxicity behavioral protocols and sufficient group sizes, studies with other thyroid hormone disrupting compounds exist which have not shown any clear behavioral effects in the offspring (Axelstad *et al.*, 2011b, 2011a, Gilbert *et al.*, 2008, 2013a; Harry *et al.*, 2014; Kodavanti *et al.*, 2010).

It seems likely that some of the apparent discrepancy between PTU and environmental chemicals can be found in the timing and severity of the fetal TH suppression. Data on the fetal T4 supply and in particular the fetal brain T4 supply are sparse, but indicate that PTU is very effective at restricting fetal T4 (Bastian *et al.*, 2012; O'Shaughnessy *et al.*).

The behavioral test employed in this study were designed to test for adverse effects, caused by a lack of thyroid hormone during development, which were irreversible, independent of concurrent effects on TH levels and which reflected altered brain function. We combined these tests with targeted gene expression profiling of the male pup PD 16 cortex. Transcript levels of TH responsive genes could potentially work as a direct molecular marker of thyroid hormone disruption in the developing brain (O'Shaughnessy *et al.*). The chosen genes have previously been implicated in PTU induced low T4 and some have been reduced dose-dependently by PTU (Bastian *et al.*, 2012; Bernal, 2017; Gil-Ibañez *et al.*, 2017; O'Shaughnessy *et al.*; Royland *et al.*, 2008). At this point in development the PFHxS-exposed pups had reduced serum T4 levels and assuming a direct correlation between serum and brain T4 levels (Gilbert *et al.*, 2013a; Morreale de Escobar *et al.*, 1988; O'Shaughnessy *et al.*; Porterfield *et al.*, 1993; Ruiz de Oña *et al.*, 1988; Schwartz *et al.*, 1997) levels it follows that thyroid hormone mediated gene expression in the cortex should be reduced. However, T4 levels were down to 55% of controls, close to the minimal

reduction required for effects on gene expression (O'Shaughnessy *et al.*), thus the effects on T4 by PFHxS may not have been above the limit of detection. Another possibility is that compensatory mechanisms lead to sufficient T4 levels in the developing pup brains so that cortical gene expression was indeed not altered, or simply that other genes than the ones examined here were affected by PFHxS exposure.

Sensitivity of behavioral studies in rats and consequences to human health

Adverse effects on human brain development have been shown in the children of women who fall within the low-normal range of T4 (Berbel *et al.*, 2009; Gyllenberg *et al.*, 2016; Haddow *et al.*, 1999; Henrichs *et al.*, 2010; Korevaar *et al.*, 2016; Román *et al.*, 2013). These results indicate that chemically induced changes to human serum T4 levels, during pregnancy, is a potential threat to human brain development. However, the testing of chemicals for effects on the developing nervous system relies on rather simple behavioral tests of rodents that do not appear to detect functional effects after more modest thyroid hormone disruption. Indeed, in humans, it is especially functional parameters as IQ and language which are affected after developmental hypothyroxinemia.

Effects by low doses and effects on sex differentiation of the brain

In addition to the marked disruption of thyroid hormones by PFHxS we found effects by PFHxS at the lowest tested dose, at 0.05 mg/kg. Although this is not correlated to the effects in thyroid hormones that were seen at higher concentrations, the effects on learning and memory at the lowest dose in both female and male adult offspring are concerning for primarily two reasons: First, this dose is far lower than the lowest tested dose would have been in a traditional developmental toxicology study design and hence, effects would have been overlooked, as the same effects where not seen at 5 and 25 mg/kg. Second, if effects are indeed present at 0.05 mg/kg in the rat, there is a much narrower safety margin between the current human exposure and what would be considered safe levels and a bigger risk

that some humans are exposed at levels that could be affecting them. This is especially true, if mixture toxicity is taken into account (Axelstad *et al.*, 2014; Christiansen *et al.*, 2012; Hass *et al.*, 2017; Howdeshell *et al.*, 2017; Kortenkamp, 2014) and we assume most perfluorinated compounds to affect the thyroid hormone system in similar ways. Such assumptions seems warranted based on both the chemical and toxicological similarities between perfluorinated compounds and the existence of the possibility for mixture effects on serum T4 levels (Crofton *et al.*, 2005; Flippin *et al.*, 2009; Gomis *et al.*, 2015; Lau *et al.*, 2007; Ren *et al.*, 2016).

Other effects not clearly correlated to TH-disruption but related to both the concerns for low-dose and mixture toxicity are the effects on activity levels in the young adult off-spring. Here, an inherent sex-difference in the rat brain had disappeared at both the low-dose and higher doses of PFHxS in combination with the background EDmix. These effects appear to be due to increased activity in the males and reduced activity in the females, i.e. the males have been feminized and the females have been masculinized. These results further supports our previous studies of the sex hormone disrupting effects of a combination of PFHxS and EDmix exposure (Ramhøj *et al.*, 2018), where we found weak signs that a combination effect of PFHxS and the EDmix was present as low as at 0.05 mg/kg PFHxS.

Since the low-dose effects observed do not follow a traditional dose-response relationship, chance findings are a possibility. Further studies confirming our observations and further characterizing the dose-response relationships for effects on sex differentiation and behavior are of great interest and could move the field forward by both expanding our understanding of these hard to study non-monotonic effects and improve our chances of safeguarding human health in the future.

Conclusion

PFHxS reduces T3 and T4 in developmentally exposed dams and pups, but does not appear to activate the HPT-axis up to 25 mg/kg bw/day. The TH disruptions did not correlate with effects on behavior and learning and memory, rather the effects suggest that PFHxS disrupted sexual differentiation of the brain rather than caused thyroid hormone mediated effects.

Human data show that T4 reductions during perinatal brain development can cause measurable reductions in child IQ. However, to regulate the use of thyroid hormone disrupting chemicals the current chemicals legislation is dependent upon the presence of a plausible link between a mode of action causing a physiologic effect and an adverse effect in animal studies. But, as we do not have sensitive tests for adverse effects of developmental hypothyroxinemia/hypothyroidism we risk not adequately protecting the developing brain in children. Therefore, to protect individuals and our societal collective cognition, we suggest using serum T4 as a marker of adverse effects until more sensitive tests are developed.

Acknowledgments

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5 Manuscript III

Developmental Immunotoxicity of Perfluorohexane Sulfonate (PFHxS) – a Study Report

Ramhøj L, Hass U, Madsen CB. 2018, *Study report.*

Developmental Immunotoxicity of Perfluorohexane Sulfonate (PFHxS)

– A Study Report

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Introduction

Integrity of the immune system is fundamental for health throughout life. However, associations between impaired immune function and serum levels of poly- and perfluoroalkylic substances (PFAS) have been seen in epidemiologic studies. The effects include asthma, susceptibility to infection, and poor long-term protection after vaccination (Dong *et al.*, 2013; Goudarzi *et al.*, 2017; Granum *et al.*, 2013; Looker *et al.*, 2014; Mogensen *et al.*, 2015; Timmermann *et al.*, 2017; Zhou *et al.*, 2017). Especially correlations between serum antibody levels in response to childhood vaccinations in Faroese children seem strong (Grandjean *et al.*, 2012, 2013, 2016; Mogensen *et al.*, 2015). There is an inverse relationship between levels of several PFAS, in either maternal serum during pregnancy or in serum of the children at age 5 and 7, and serum vaccine antibody responses to tetanus and diphtheria vaccinations at age 5, 7 and 13. As these effects also results in an increased odds ratios for having no protective effect of the childhood vaccinations, the epidemiological studies are highly concerning (Grandjean *et al.*, 2012). Studies in animals of compounds as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have confirmed their potential immunotoxic effects but animal studies of PFHxS are missing (Dewitt *et al.*, 2008; FSANZ, 2016; Keil *et al.*, 2008; Peden-Adams *et al.*, 2008; Yang *et al.*, 2002). In developmental toxicity studies of PFHxS we wanted to investigate the potential for developmental immunotoxicity of PFHxS on functional immune endpoints: antibody response to vaccination, delayed type hypersensitivity and avidity. To discern between effects induced by developmental exposure through the dam and effects caused by

direct exposure of young animals, these two exposure scenarios were investigated in separate studies.

Materials and methods

Animals and treatment

Two developmental toxicity studies were performed with time-mated Wistar (HanTac: WH) rat dams (Taconic, Denmark). The study designs have been described in details in Ramhøj et al., (2018, see Study 1 and 2). Briefly, in Study 1 the immunotoxicity part of the study consisted of 3 groups of 8 animals dosed with vehicle (corn oil), 25 or 45 mg/kg bw/day of PFHxS (Perfluorohexane Sulfonate (PFHxS) purity >98 %, Sigma-Aldrich (Tridecafluorohexane-1-sulfonic acid potassium salt, CAS-No: 3871-99-6, lot #BCBC3545V). In Study 2 the immunotoxicity part of the study comprised 4 groups of 20 animals dosed with PFHxS; 0.05, 5 and 25 mg/kg bw/day. Corn oil (Sigma-Aldrich) was used as vehicle and dosing in the control group.

Animal were received on gestation day (GD) 3 of pregnancy and dosing of the dams was performed daily by oral gavage from GD 7 through postnatal day 22 (PND), except the day of delivery. The expected day of delivery (GD 23) was termed PND 1 for the pups. Hence, the age of the pups was related to time of mating rather than day of birth.

From the day of arrival the housing standard controlled environmental conditions were: Reversed light/dark cycles of 12 hours (light from 9 pm-9 am), humidity 55 ± 5 %, temperature at 21 ± 1°C, and ventilation changing air ten times per hour. All animals were

fed ad libitum on a standard diet with Altromin 1314 (soy and alfalfa-free, Altromin GmbH, Lage, Germany) and were provided ad libitum acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast).

Weaning was performed on PND 22. In Study 1, 8 males and 8 females per group were weaned (representing 5-7 litters, not adjusted for litter effects in the analysis). In Study 2, up to one male and one female pup from each litter were weaned and housed pair- or triple-wise with animal(s) of the same sex and group, and dams were sacrificed. These cohorts of weaned offspring were used for functional immune testing as described below.

A study with the positive control compound cyclophosphamide was conducted with 40 young adult Wistar rats from Charles River, Germany. Animals were received on PND ~24 randomly allocated to 5 groups of 8 animals with equal weight distributions and housed pairwise under standard conditions as in Study 1 and 2. Animals were dosed once daily from PND ~30 with vehicle (saline), 5 mg PFHxS/kg bodyweight (bw)/day in corn oil or cyclophosphamide at 1, 3 or 10 mg/kg bw/day. Immunizations were performed as described below. The high dose of cyclophosphamide caused weight loss and toxicity in the animals so the dose was halved to 5 mg/kg bw/day on PND ~48 and stopped at PND ~51. 1 animal was killed ~49 because of ill health, one died ~51 and 3 were euthanized on PND ~53, toxicity persisted even after reduction of exposure, so the remaining animals from the high dose group were sacrificed on PND ~57.

The animal experiments were carried out at the DTU National Food Institute facilities. Study 1 and 2 in Mørkhøj, and the cyclophosphamide study in Lyngby Denmark. Ethical

approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2015-15-0201-00553 C3. The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Autopsies, immunizations and blood samples

Procedures were equal in Study 1 and 2 unless specified. Necropsy took place on PND16/17 and PND 22 where up to one male and one female pup per litter was macroscopically evaluated. On PND 16/17 thymus, spleen and popliteal lymph nodes were excised and weighed. On PND 22 spleen and thymus were excised and weighed.

Weaned offspring were immunized twice (14 days apart) with 0.3 mg KLH intraperitoneally (i.p.) (200 µl of 1.5 mg/ml KLH) (Hemocyanin from Megatura crenulata (keyhole limpet) from Sigma-Aldrich, H7017-20 mg, Lot 065M4834V). In Study 1, Immunizations were performed on PND 23 and PND 37 and in Study 2 on PND 29 and PND 43. In the cyclophosphamide study immunizations took place on PND ~44 and PND ~58.

Blood samples were taken from the tongue vein without anesthesia in Eppendorf tubes. In Study 1, sampling took place on PND 28, 37 (before immunization) and PND 43, in Study 2, on PND 34, PND 43 (before immunization) and PND 48. In the cyclophosphamide study on PND ~49, PND ~58 (before immunization) and PND ~63. Blood was allowed to clot at room temperature for 1 h and stored in refrigerator (5℃) overnight. After overnight

storage clots were removed and tubes centrifuged at 3000 rpm for 20 min and the clear serum supernatant stored at -20℃ until analysis.

In Study 1 and 2, necropsy of all animals took place on PND 44 and PND 50, respectively. Thymus, spleen, popliteal lymph nodes, left medial mediastinal lymph node, liver and adrenals were excised and weighed. In the cyclophosphamide study necropsy was performed on PND ~63 with excision and weighing of liver, thymus, and spleen.

Specific IgG1 and IgM

Levels of specific serum IgG1 and IgM was determined via enzyme-linked immuno sorbent Assay (ELISA). 96-well plates were coated overnight with 5 µg KLH /mL in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6). A positive and negative control serum pool was included on each plate to check assay reproducibility. Plates were incubated (1h) with serially diluted sera in PBS (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4*2H_2O$, 1 mM KH_2PO_4, pH 7.2) containing 0.01% Tween (PBS-T). Then, incubated (1h) with anti-IgG1 (1:20000) or anti-IgM (1:16000) antibodies in PBS-T (anti-IgG1: horseradish peroxidase (HRP)-mouse-anti-rat IgG1(3060-05), anti-IgM: HRP-goat-anti-rat IgM (3020-05). Both from Southern Biotech, Birmingham, AL, USA). Development was performed with TMB-one (Kem-En-Tec, Copenhagen, Denmark) for 12 min in the dark and stopped with 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments). Levels of IgG1 and IgM are expressed as titers on a Log2-scale with cutoff determined

according to the negative control for IgG1 and IgM assays. The positive control was used to check reproducibility of the assay.

Avidity

For measurement of the strength of binding between KLH and IgG1 antibodies a thiocyanate inhibition ELISA based on the method described by El-Khouly et al. was conducted (El-Khouly *et al.*, 2007). Overall assay, coating and reagents as described for above for the specific IgG1 assay. Serum samples were diluted in PBS-T to give an OD between 0.5 and 1, 50 µl added to each well and incubated (1h). Then, incubation (30 min) with potassium thiocyate (KSCN, Sigma, St. Louis, MO, USA) in increasing concentrations (0, 0.1, 0.2, 0.5, 1 and 2 M in PBS-T, quadruplicate determinations for each concentration). For detection, incubation (1h) with anti-IgG1 (see specific IgG1 assay). Reaction was visualised by adding 100 µL/well of TMB-one for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4.

Under the given assay conditions, it was determined that KSCN did not influence the binding of antigen to the plates.

Avidity is expressed as the concentration of KSCN inhibiting 50% (IC_{50}) of the antigen-antibody interaction. The higher the concentration, the stronger is the interaction between antigen and antibody.

Delayed type hypersensitivity

In Study 1 and 2, the day prior to necropsy (PND 43 and 49, respectively), animals were mildly anesthetized with hypnorm/dormicum. One ear was challenged intradermally with injection of 20 µl of a 5 mg/ml KLH solution and the other ear was injected with 20 µl 0.9% sterile saline solution. To avoid bias, left and right ear was alternated to receive the challenge. Necropsy was performed approximately 24h after the ear injection. Immediately after decapitation the thickness of both ears was measured with a micrometer caliper at the site of injection and a biopsy was taken with a 6 mm biopsy puncture and weighed. The saline ear served as control ear of each animal and the change in ear thickness and weight was calculated by subtracting the thickness/weight of the saline ear from the KLH challenged ear.

Statistical Analysis

Data from continuous endpoints were checked for normal distribution and homogeneity of variance and then analyzed by analysis of variance (ANOVA). If assumptions were not fulfilled, data was transformed accordingly. Body weight was included as covariates in data analyses when considered as relevant. Antibody titer levels were analyzed by Kruskal-Wallis. Litter effects were accounted for by not assessing more than one pup per sex per litter in all analyses except the weaned cohort in Study 1. Here, due to limited group sizes up to two siblings per sex were included resulting in n = 8 representing 5-7 litters. The global Type I error was controlled by the two-tailed Dunnett's test or Dunn's multiple

comparison, with the alpha level for statistical significance always set to 5%. SAS Enterprise Guide 4.3 (2010), SAS Institute Inc, Cary, NC 27513, USA, and GraphPad Prism 5.00 (2007) GraphPad Software, Inc, La Jolla, CA 92037, USA were used for statistical analyses.

Results

Organ weights

Organ weights from Study 1 are shown in Table 1. On PND 16 male offspring popliteal lymph node weight was reduced nearly 50% at both 5 and 25 mg/kg PFHxS ($p = 0.0277$ and $p = 0.0205$, respectively) but no effects were seen in female offspring. There were no effects, in any sex, on bodyweight, thymus and spleen. At PND 44 the only effect was reduced adrenal gland weight in 5 mg/kg PFHxS male offspring ($p < 0.001$).

In Study 2 no statistically significant effects on body weight, popliteal lymph nodes, spleen and thymus for either male or female offspring PND 16/17 were found (Table 2). Body weight, liver and adrenal gland weights from PND 16/17 Study 2 were reported previously (Ramhøj *et al.*, 2018). The only effects observed was an increase in female liver weight and in male adrenal gland weight in the 25-PFHxS group. At autopsy of the immunized cohort PND 50, no effects were found on body weight, adrenal glands, liver or immunological organs (Table 2).

In the cyclophosphamide study, cyclophosphamide had no effects on spleen, thymus or liver weight (Table 3). Likewise, there were no statistically significant effects on body weight PND 64 in the two cyclophosphamide groups that completed the study. The high dose group (10 mg/kg bw/day) was terminated early due to toxicity and body weight loss of approximately 20% compared to control. In the 5 mg/kg PFHxS group there were no effects on body weight, spleen or thymus weight, but the weight of the liver was increased with approximately 25% compared to control ($p < 0.001$).

Table 1. Absolute organ weights Study 1 (PFHxS range-finding study). Analysis performed with body weight included as covariate.

	Control	25-Px	45-Px
PND 16 male, n	5	5	5
Body weight[a] (g)	35.8 ± 5.8	30.5 ± 1.2	33.4 ± 9.3
Popliteal lymph nodes (mg)	3.9 ± 0.8	**2.3 ± 0.7***	**2.4 ± 0.8***
Spleen (mg)	143.5 ± 47.7	106.6 ± 25.8	128.8 ± 58.8
Thymus (mg)	133.5 ± 34.7	106.3 ± 8.9	108.9 ± 50.7
PND 16 female, n	5	6	7
Body weight[a] (g)	33.7 ± 8.0	31.8 ± 3.2	32.7 ± 8.5
Popliteal lymph nodes (mg)	3.0 ±1.0	2.9 ± 0.6	3.1 ± 0.9
Spleen (mg)	142.1 ± 75.5	129.9 ± 32.7	146.7 ± 61.1
Thymus (mg)	119.9 ± 51.2	118.1 ± 23.3	122.9 ± 32.1
PND 44 male, n (litters)	8 (5)	8 (6)	8 (7)
Body weight (g)	166.4 ± 15.8	150.3 ± 20.8	154.1 ± 18.7
Popliteal lymph nodes (mg)	8.6 ± 1.4	10.3 ± 3.5	10.0 ± 3.1
Left lateral mediastinal lymph node (mg)	4.3 ± 2.7	3.2 ± 1.3	3.3 ± 1.7
Spleen (mg)	628.0 ± 87.3	643.6 ± 93.7	622.1 ± 106.2
Thymus (mg)	505.8 ± 74.0	508.5 ± 64.2	522.0 ± 112.3
Adrenals (mg)	43.0 ± 6.8	**35.5 ± 4.2 ***	37.9 ± 4.8
Liver (g)	7.23 ± 0.63	6.74 ± 0.80	7.27 ± 1.20
PND 44 female, n (litters)	8 (5)	8 (6)	8 (7)
Body weight (g)	133.1 ± 12.9	146.1 ± 25.7	136.8 ± 14.0
Popliteal lymph nodes (mg)	8.5 ± 1.8	9.2 ± 1.2	8.0 ± 1.0
Left lateral mediastinal lymph node (mg)	2.6 ± 1.4	3.7 ± 1.1	3.7 ± 2.1
Spleen (mg)	467.5 ± 48.9	490.3 ± 72.1	473.8 ± 63.7
Thymus (mg)	466.9 ± 48.4	488.9 ± 60.2	434.6 ± 96.0
Adrenals (mg)	42.6 ± 7.9	43.5 ± 5.1	45.3 ± 9.1
Liver (g)	5.45 ± 0.51	5.64 ± 0.73	6.01 ± 0.86

Mean ± SD

[a]Body weight reported previously (Ramhøj *et al.*, 2018). Included here for reader discretion.

Table 2. Absolute organ weights Study 2 (PFHxS main study).
Analysis performed with body weight included as covariate.

	Control	0.05-Px	5-Px	25-Px
PND 16 male, n	16	14	14	14
Body weight[a] (g)	33.7 ± 4.8	34.1 ± 5.6	32.4 ± 3.5	30.5 ± 4.7
Popliteal lymph nodes (mg)	3.0 ± 0.5	3.5 ± 0.9	2.6 ± 0.8	2.4 ± 0.6
Spleen (mg)	135.7 ± 41.1	127.7 ± 38.6	117.6 ± 33.8	103.7 ± 25.2
Thymus (mg)	122.3 ± 23.2	135.5 ± 39.4	117.6 ± 20.6	110.9 ± 23.2
PND 17 female, n	16	11	16	16
Body weight[a] (g)	35.7 ± 5.6	33.9 ± 4.9	34.5 ± 5.1	33.3 ± 4.6
Popliteal lymph nodes (mg)	3.2 ± 1.0	3.3 ± 0.9	3.3 ± 0.7	3.0 ± 0.8
Spleen (mg)	145.5 ± 52.3	122.1 ± 26.3	128.8 ± 6.6	128.6 ± 34
Thymus (mg)	142.7 ± 25.3	124.6 ± 34.0	135.1 ± 26.8	125.5 ± 29.1
PND 22 male, n	17	12	15	14
Body weight[a] (g)	51.0 ± 6.3	50.8 ± 8.0	50.3 ± 5.4	48.6 ± 6.5
Spleen (mg)	246.0 ± 77.1	254.5 ± 93.2	229.5 ± 53.4	223.8 ± 75.9
Thymus (mg)	207.4 ± 30.1	208.8 ± 46.0	203.0 ± 32.9	193.8 ± 33.4
PND 22 female, n	17	11	16	15
Body weight[a] (g)	49.6 ± 4.6	49.4 ± 6.9	49.5 ± 5.8	49.2 ± 6.0
Spleen (mg)	253.1 ± 67.3	229.8 ± 58.2	233.8 ± 48.7	230.7 ± 75.2
Thymus (mg)	187.8 ± 36.3	215.3 ± 50.0	201.5 ± 28.8	198.1 ± 28.7
PND 50 male, n	16	14	16	16
Body weight (g)	192.6 ± 15.4	201.0 ± 19.0	196.6 ± 15.2	192.1 ± 14.8
Popliteal lymph nodes (mg)	11.8 ± 6.0	11.7 ± 5.0	10.2 ± 3.6	10.7 ± 4.0
Left lateral mediastinal lymph node (mg)	4.5 ±3.7	4.6 ± 1.6	4.7 ± 3.1	4.6 ± 2.8
Spleen (mg)	685.5 ± 88.6	714.0 ± 121.0	698.0 ± 98.5	662.4 ± 97.4
Thymus (mg)	552.4 ± 78.6	616.1 ± 97.2	560.1 ± 115.4	568.9 ± 74.5
Adrenals (mg)	41.2 ± 6.2	42.4 ± 8.2	40.5 ± 7.0	41.8 ± 6.0
Liver (g)	8.76 ± 0.88	9.25 ± 1.30	8.86 ± 0.87	8.54 ± 1.39
PND 50 female, n	16	14	16	16
Body weight (g)	150.7 ± 8.2	160.4 ± 13.0	168.3 ± 34.3	153.9 ± 12.3
Popliteal lymph nodes (mg)	9.8 ± 3.4	11.2 ± 3.9	8.9 ± 3.2	9.8 ± 3.0
Left lateral mediastinal lymph node (mg)	4.5 ± 3.0	3.3 ± 1.1	3.4 ± 1.7	4.6 ± 2.9

Spleen (mg)	471 ± 57.4	505.1 ± 69.7	490.2 ± 67.5	482.9 ± 77.9
Thymus (mg)	444.0 ± 80.7	494.9 ± 93.4	452.4 ± 54.9	448.3 ± 79.6
Adrenals (mg)	48.2 ± 5.2	50.3 ± 5.8	50.4 ± 6.8	49.2 ± 5.2
Liver (g)	6.44 ± 0.47	6.88 ±0.67	6.32 ± 0.50	6.55 ± 0.80

Mean ± SD

[a]Body weight reported previously (Ramhøj *et al.*, 2018). Included here for reader discretion.

Table 3. Absolute organ weights cyclophosphamide study.
Analysis performed with body weight included as covariate.

	Control	**5-Px**	**CY 1 mg**	**CY 3 mg**
PND ~64 male, n	8	8	8	8
Body weight (g)	350.0 ± 19.4	348.2 ± 22.0	364.08 ± 31.1	333.6 ± 11.7
Spleen (mg)	866.0 ± 134.8	902.0 ± 121.2	1002.4 ± 136.5	731.1 ± 95.1
Thymus (mg)	599.4 ± 80.2	614.5 ± 156.9	674.8 ± 100.0	578.9 ± 75.0
Liver (g)	13.61 ± 0.90	**17.06 ± 1.53 *****	15.03 ± 1.83	13.33 ± 1.21

Mean ± SD

***$p < 0.001$ compared to control

Antibody responses

Developmental exposure to PFHxS

Antibody levels for Study 1 are depicted in Fig. 1. Primary IgM titers in Study 1 were quite low but detectable in most animals, they had risen in the secondary response in most animals, but no statistically effects were observe in either sex. Likewise, the primary IgG1 response was quite restricted with signs of increased response in the 45 mg/kg PFHxS males. Again, the secondary titers were increased compared to the primary response. In the male offspring some low responders were observed in the control group, and the 45 mg/kg group had serum IgG1 levels significantly elevated compared to controls. For the females, the same tendency of increase was apparent at 25 mg/kg PFHxS, however at 45 mg/kg four animals were non-responders, hence reducing mean value below control level. Overall, interpretation of results is significantly hampered by a very large variation within groups.

In an attempt to reduce variation, potentially caused by increased stress after weaning of the offspring, time of immunization in Study 2 was postponed 5 days to PND 29. No statistically significant effects were found in offspring in this study (Fig. 2). Primary IgM responses show a

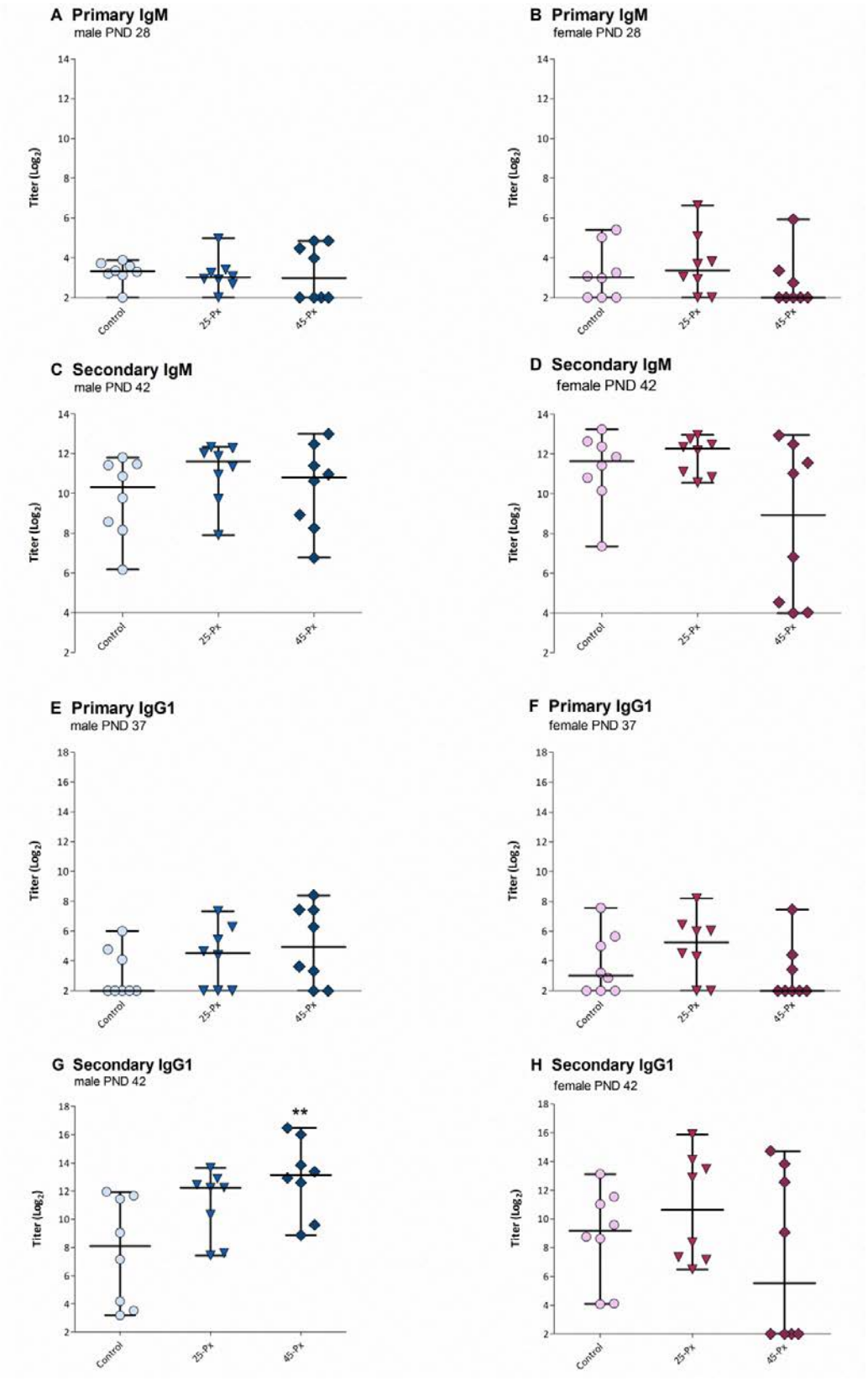
A Primary IgM
male PND 28
B Primary IgM
female PND 28
C Secondary IgM
male PND 42
D Secondary IgM
female PND 42
E Primary IgG1
male PND 37
F Primary IgG1
female PND 37
G Secondary IgG1
male PND 42
H Secondary IgG1
female PND 42
Titer (Log2)
Control
25-Px
45-Px
**

Fig. 1. Specific serum IgM and IgG1 responses after immunizations with KLH and developmental exposure to PFHxS, Study 1. Immunizations performed on PND 23 and PND 37. PND: postnatal day. Individual animals represented by symbols. Lines and whiskers represent median ± range. n = 8 representing 5-7 litters.

high variability in both sexes whereas the variation is lower in the secondary and IgG1 responses, although still with some animals being poor responders and primary IgG1 levels in high dose males (25 mg/kg) being quite variable. Generally, antibody levels are increased compared to Study 1 and means are quite similar over exposure groups. The distribution pattern potentially indicating that maximal titer levels have been reached in the animals.

Cyclophosphamide and direct exposure to PFHxS

The cyclophosphamide study included a group of young males dosed with PFHxS. In this group there was no effect on IgM or IgG1 responses (Fig. 3). Cyclophosphamide had no statistically significant effect on primary IgM, and primary and secondary IgG1 responses. However, only primary IgM titers are available from the high dose group (10 mg/kg) as the animals were terminated prematurely for welfare reasons. No animals in the high dose group had a measurable primary IgM titer while 3 controls where above detection limit and 5, 4 and 3 animals were above detection limits in the 5-Px, CY 1 mg and CY 3 mg groups, indicating an impaired primary IgM response at 10 mg/kg Cyclophosphamide. The primary IgG1 response appeared to be dose-dependently increased by cyclophosphamide, however not statistically significant and the trend was less pronounced in the secondary response.

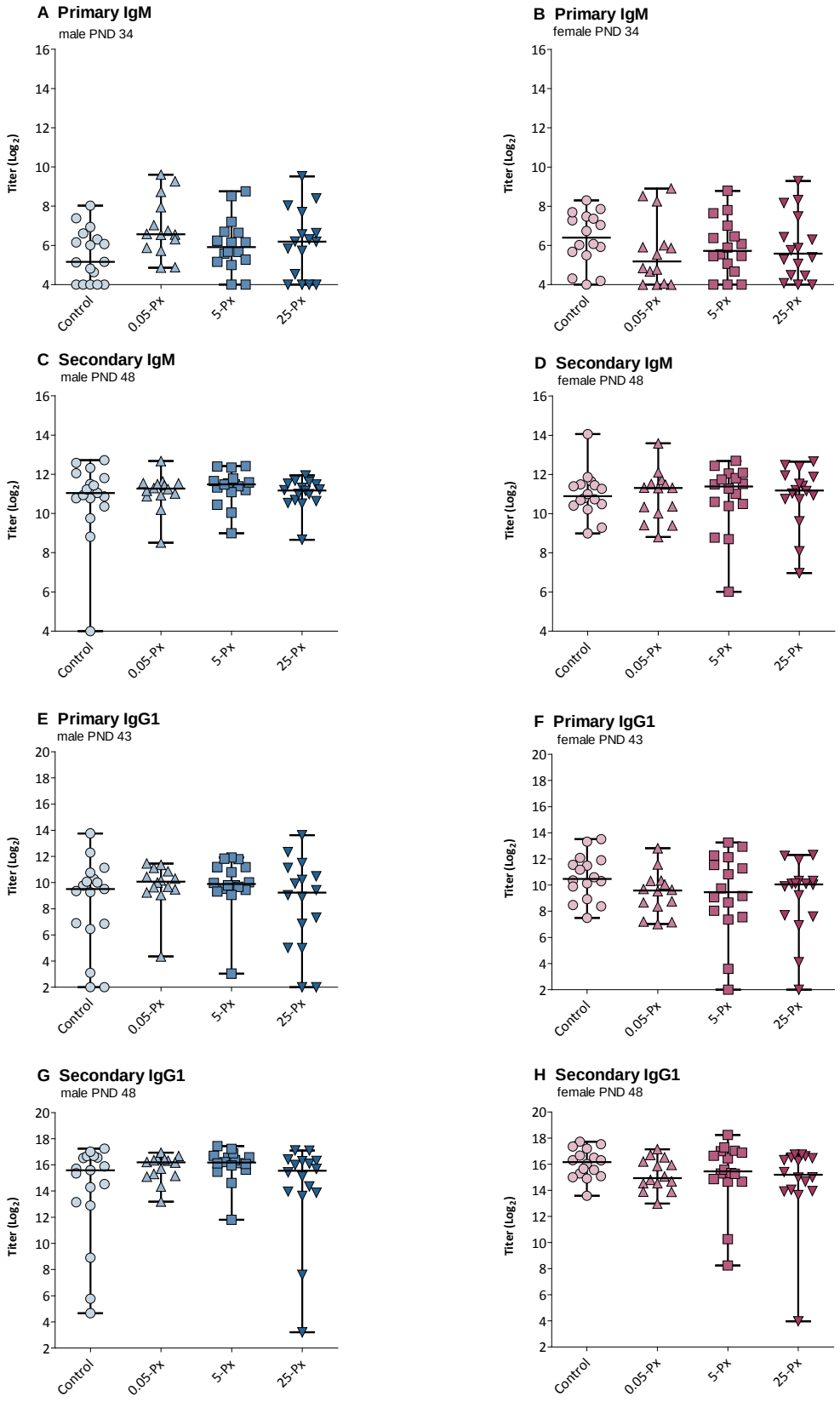
A Primary IgM
male PND 34
B Primary IgM
female PND 34
C Secondary IgM
male PND 48
D Secondary IgM
female PND 48
E Primary IgG1
male PND 43
F Primary IgG1
female PND 43
G Secondary IgG1
male PND 48
H Secondary IgG1
female PND 48
Titer (Log2)
Control
0.05-Px
5-Px
25-Px

Fig. 2. Specific serum IgM and IgG1 responses after immunizations with KLH and developmental exposure to PFHxS, Study 2. Immunizations performed on PND 29 and PND 43. PND: postnatal day. Individual animals represented by symbols. Lines and whiskers represent median ± range. n = 14-16.

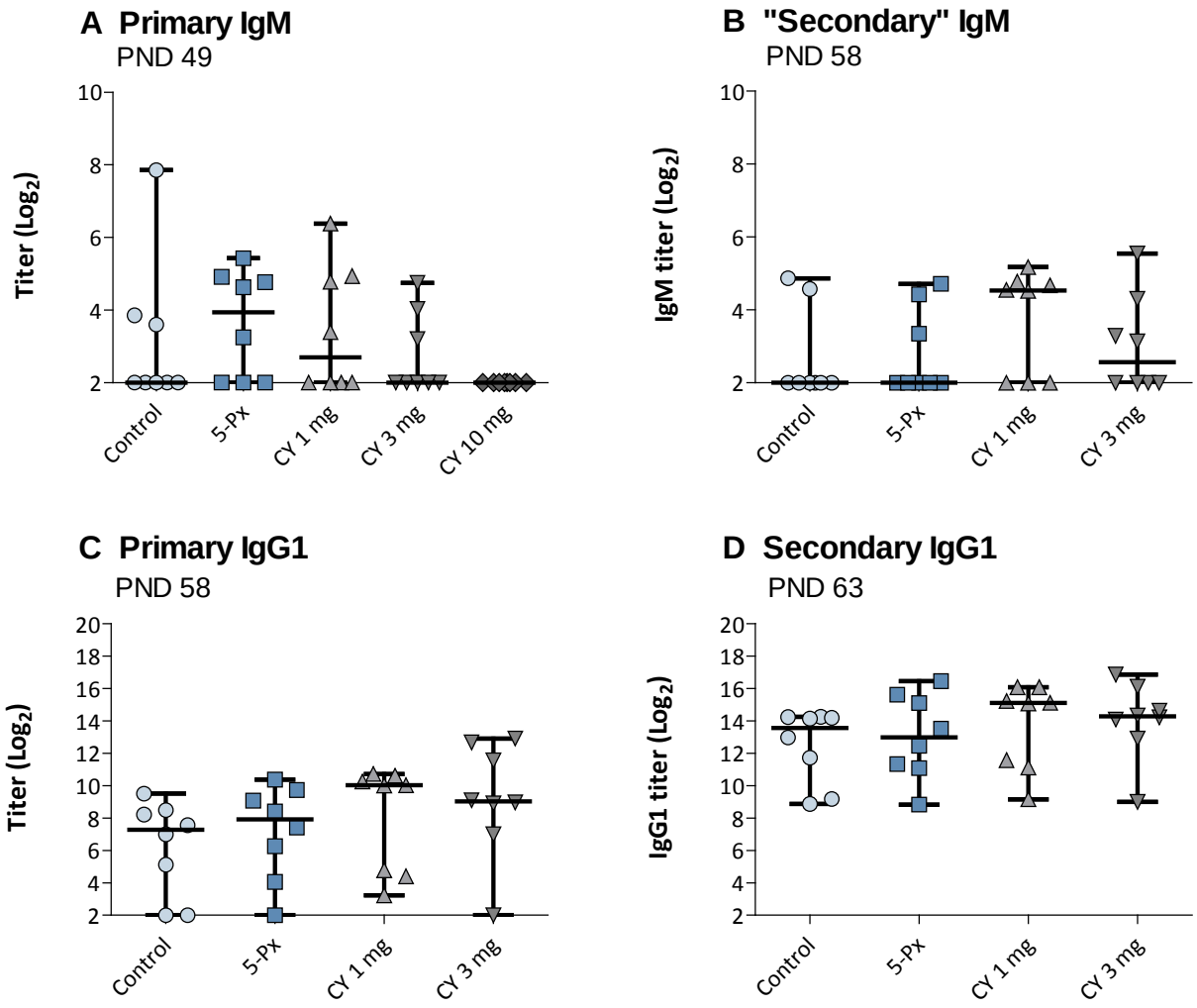

Fig. 3. Specific serum IgM and IgG1 responses after immunizations with KLH during exposure to PFHxS or Cyclophosphamide from PND 30, cyclophosphamide study. Immunizations performed on PND 44 and PND 58. PND: postnatal day. Individual animals represented by symbols. Lines and whiskers represent median ± range. n = 8, all males.

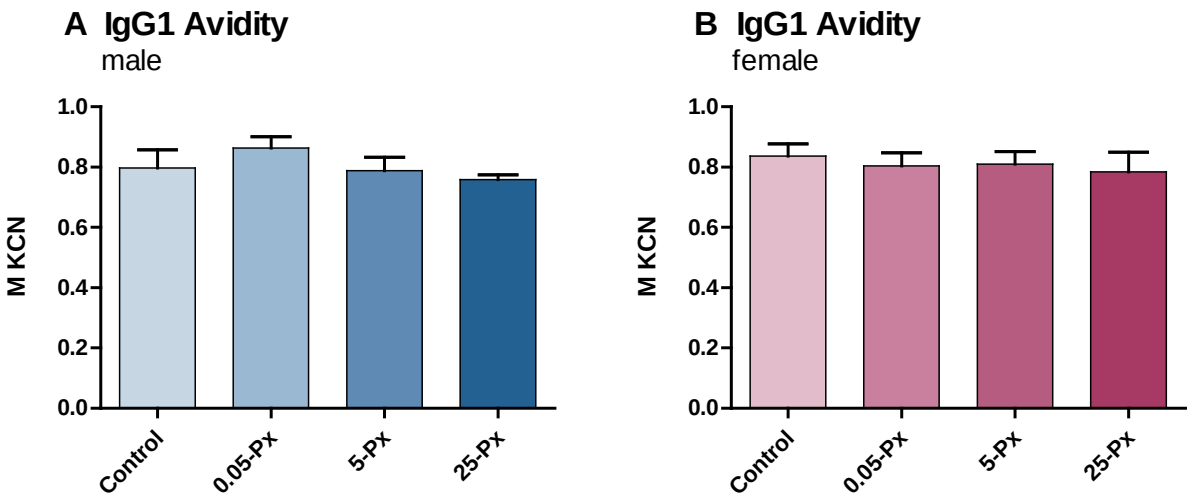

Fig 4. IgG1 Avidity in male and female offspring after immunizations with KLH and developmental exposure to PFHxS, Study 2. Immunizations performed on postnatal day (PND) 29 and PND 43 and avidity determined on PND 50. Data shown as mean + SEM. n = 14-16.

Avidity

No statistically significant effects were found on avidity of IgG1 antibodies in the PND 50 offspring from Study 2 (Fig. 4).

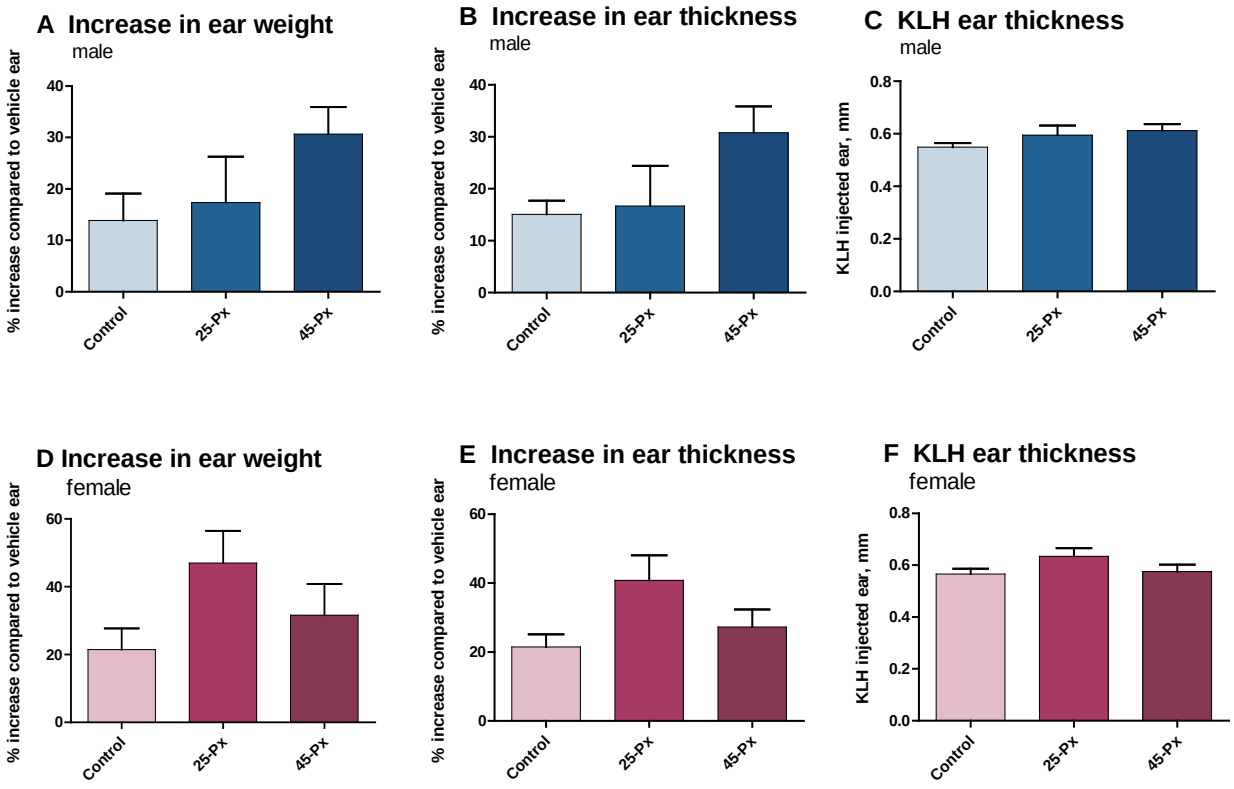

Fig 5. Delayed type hypersensitivity in male and female offspring after immunizations with KLH and developmental exposure to PFHxS, Study 1. Immunizations performed on postnatal day (PND) 23 and PND 37 and challenge with KLH and saline on PND 34 followed by ear measurements 24h later. Data shown as mean + SEM. n = 8 representing 5-7 litters.

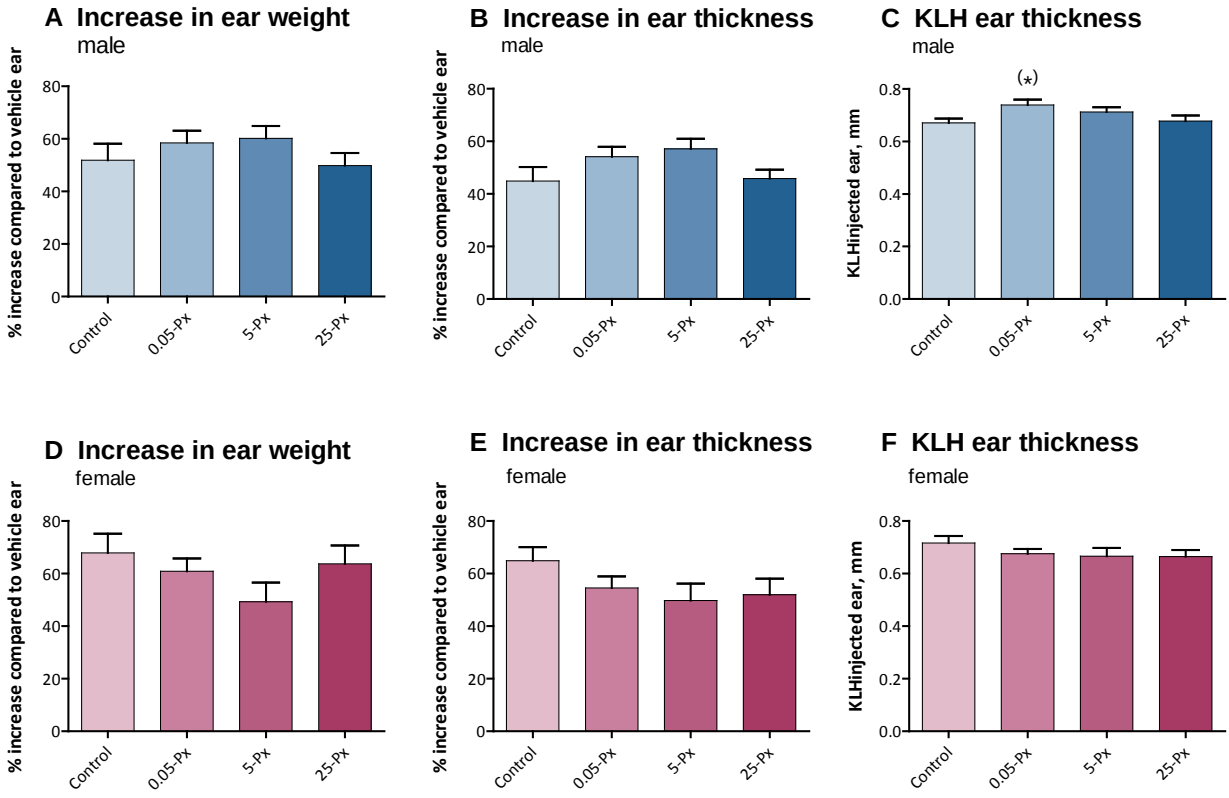

Figure 6. Delayed type hypersensitivity in male and female offspring after immunizations with KLH and developmental exposure to PFHxS, Study 2. Immunizations performed on postnatal day (PND) 29 and PND 43 and challenge with KLH and saline on PND 49 followed by ear measurements 24h later. Data shown as mean + SEM. n = 14-16.

Delayed Type Hypersensitivity

No statistically significant effects were found on delayed type hypersensitivity as measured via ear thickness and ear biopsy weight after challenge with KLH (Fig. 5, Study 1 and Fig. 6 ,Study 2). The measure showed high variability, hence low power. In both studies strong correlations between the 3 measures of ear thickness (DTH) were observed. The results

also revealed correlations between primary and secondary IgG1 responses and DTH suggesting activation of both Th1 and Th2-type responses after the immunization with KLH.

Discussion

The functional immunotoxic potential of PFHxS in animal studies is unknown, whereas multiple epidemiological studies have found associations with effects in humans (Dong *et al.*, 2013; Goudarzi *et al.*, 2017; Granum *et al.*, 2013; Looker *et al.*, 2014; Mogensen *et al.*, 2015; Timmermann *et al.*, 2017; Zhou *et al.*, 2017). The studies presented here aimed at investigating a relationship between developmental immunotoxicity in animals and exposure to PFHxS. However a high variation in study parameters and the failure of a positive control study precludes conclusions based on the studies. In the following we will discuss the model, the few results we can draw conclusions on, and suggest improvements to design of future studies.

Positive control study with cyclophosphamide

The positive control study with cyclophosphamide suffers from multiple severe limitations, most noticeably the lack of effect on serum antibody titers, effectively rendering our

studies useless. Regrettably, for practical reasons, the study had to be performed after the studies of PFHxS and it was carried out in a new facility at DTU Lyngby (although still with similar standard conditions in the new facility). This timeline is the reason that the animal model was not adjusted accordingly and validated again after the failed cyclophosphamide study. No apparent explanation exist for the lack of effects on antibody titers after exposure to cyclophosphamide where others have found effects (Hou *et al.*, 2007; Kawai *et al.*, 2013; Loveless *et al.*, 2007), although the literature does suggest that positive control studies in immunotoxicology may not always produce significant results in all laboratories (section 2.6.2) In one of the validation studies Richter-Reichhelm et al.,(1995) compared five labs in a 28-day study of cyclosporine A with additional immunotoxicity endpoints (Richter-Reichhelm *et al.*, 1995). Interestingly, at the highest dose, specific IgG was reduced in females but *increased* in males. In two labs out of five, specific IgM titers were decreased in male rats. These results seem to resemble our findings in the cyclophosphamide and PFHxS range-finding study and suggest that detection of effects even with positive control compounds may not always be as expected. Furthermore, it indicates the importance of sex in immunotoxicology studies and shows that the different endpoints reflect effects on different components of the immune system. These components may all be affected in dissimilar ways and examination of them all could be necessary to correctly predict the immunotoxic potential of a given compound.

Other studies suggests that cyclophosphamide may not always function as an overall immune suppressing agent but that certain circumstances and dosing schemes rather result in an immune potentiation, possibly even increasing immune responses, as our cyclophos-

phamide study could also suggest (Brode *et al.*, 2008; Sistigu *et al.*, 2011; White *et al.*, 2007). On the other hand we simply may not have been able to use a dose high enough to aquire enough sensitivity; the highest dose should cause a 10 % decrease in body weight (Dayan *et al.*, 1998). However, this was not the case for our studies of either PFHxS or cyclophosphamide where the highest dose group surviving until the end of the experiment had a reduction of 4.9% possibly indicating a low sensitivity of our studies (Hou *et al.*, 2007).

While unreliability and differential mechanisms may be the explanation for our negative findings, other potential sources of variation between the cyclophosphamide and PFHxS studies also deserve scrutiny and severely impair comparability between studies. Wistar rats were used for all studies, but for the cyclophosphamide study, the Danish breeder had suddenly discontinued the Wistar strain, hence the rats had to be delivered from Charles River, Germany. Therefore, the rats were from the same strain but originated from 2 different breeding populations, potential source of variation. Another potential source of variation arises in the timing of the cyclophosphamide study. While the first immunization in Study 1 and 2 took place on PND 23 and 29, respectively, the first immunization in the cyclophosphamide study took place on ~PND 38. Possibly even later as the rats ordered as PND 24 rats upon delivery weighed 90 grams on average, a bit more than the ca 55 gram they weigh at the same age when they are born in our facility.

All of the above factors may have influenced the results and contributed with variability, however, we should still have been able to detect effects in a positive control study.

Antibody responses after immunization with KLH as an animal model to detect developmental immunotoxicity

CoV

The immunization scheme to be employed in the PFHxS studies was optimized for KLH dose and immunization route in two pilot studies in young adult Wistar rats (Taconic, Denmark). A dose of 0.3 mg/kg KLH administered intraperitoneally was found optimal for detection of both increased and decreased antibody responses and on both primary and secondary IgM and IgG1 responses with coefficient of variations (CoV) between 10.4 and 16.5%. However, when this model was applied to the cohorts of pups from the developmental toxicity studies the CoVs for the antibody responses in the controls were between 7.0% and 59.8%, significantly decreasing the power and reliability of the studies.

Baseline

The absolute antibody titers vary considerably between the two PFHxS studies; whereas the titers in the primary responses of Study 1 are so low that it effectively impedes detection of immunosuppression, the levels of the secondary responses and potentially also primary IgG1 from Study 2 could have reached maximum, precluding detection of increased responses and having activated the immune response so effectively that even the detection of immunosuppression would be with low sensitivity due to the strength of the induced response (and hence high potency required to revert the induced response). This

could also (partly) be the explanation for our failed cyclophosphamide study; the KLH i.p. immunizations may have been activating the full capacity of the immune system, effectively compensating for the presumed cyclophosphamide induced immune suppression.

Changes to baseline could also be due to changes in the antigen, where subunit confirmation and sources of KLH has shown differential antigenicity (Lebrec *et al.*, 2013). The same lot of KLH was used for all studies, although the KLH for the cyclophosphamide had been stored up until the end of its shelf life.

Inter-studies variability

Direct comparison between absolute titer values from different studies should be done with caution due to possible variation in the ELISA assay employed. We tried to overcome this by using the positive control to test the stability of the assay; still, comparisons to the concurrent control group are the most reliable. In our studies this mostly gives rise to the conclusion of no effect. However, the considerable difference between the studies suggest a need for further refinement of the animal model to ensure adequate possibility for detection of both increasing and decreasing effects on the antibody responses at the different phases of both IgM and IgG1 responses. If the marked differences observed between studies is representative, it may even be necessary with different immunization protocols for different immune effects and potentially also with a concurrent positive control group in every study.

Study design

We attributed some of the variation in the range-finding study to potentially be due to increased stress in the animals who had been weaned the day prior to the first immunization; hence immunizations in the main study were delayed 5 days, also allowing for further discrimination between true developmental effects and effects due to the internal dose of PFHxS. This shift in developmental age may provide some of the explanation for the differences in response profiles in the two PFHxS studies, as it can be assumed that the immune system is more mature in the older animals studied in the main study, although both studies took place during a time of rapid change in the immune system of these pubertal rats (Boverhof *et al.*, 2014; Holsapple, 2005; Tonk *et al.*, 2010, 2012). The significance of accurate timing of immunizations should be studied further also with the prospect of reducing variation within groups.

The immune system over a lifetime

Our studies, as well as OECD guideline studies, fail to adequately test the immune system over the lifetime of the animal with termination around day 50. For the OECD guideline studies the assessed time window is even narrower with continued exposure of pups after weaning and termination day 56 (± 3 days) with immunization 4 to 5 days before (OECD, 2011). Effects on the immune system due to developmental exposure may arise later in life for example in the form of skewing of immune responses and increased risk of non-communicable diseases (Dietert *et al.*, 2009; IPCS, 2012). Immunotoxicity studies should

therefore assess these possibilities in addition to effects during development and maturation of the system (Dietert, 2014).

Additional endpoints

Maturation of antibody responses is an important functionality of the immune system. Here, both the measures of primary and secondary responses and determination of avidity of the antibodies can serve for evaluation of this part of the immune system. The level of specific antibody as measured by ELISA may not be the most sensitive endpoint for antibody response (Bøgh *et al.*, 2013; Kroghsbo *et al.*, 2011). We therefore chose to include the binding strength (avidity) of the response, as it is a measure of the maturation of the specific response. There were no effects on avidity hence on the maturation of response between groups.

Delayed type hypersensitivity was included in our PFHxS studies to expand our studies to also include a more Th1-cell dependent response. It was assessed as increase in ear thickness and weight of ear biopsy 24h after a KLH challenge. We saw a high degree of correlation between the two measures and also to IgG1 responses. This suggests an equal degree of T and B-cell activation by i.p. KLH. It is also worth noting that the high variability within groups for the DTH response results in a low power, even with large nominal effects, statistical significance is unlikely with the current setup. Hence, nominal effects on DTH could function as circumstantial additional evidence to support a significant effect on antibody responses.

Conclusion

Effects on the immune system after exposure to PFHxS were scarce; however a faulty study design impedes conclusions on especially functional immune endpoints, which in theory should be the most sensitive endpoint in immunotoxicology. At a high dose (45 mg/kg) PFHxS may reduce immune organ weights, but this could not be replicated in the main study investigating doses up to 25 mg/kg. Hence further studies of immunotoxicity of PFHxS are warranted.

Other studies have shown that perfluorinated compounds are a threat to healthy immune function amongst humans and animals. While the evidence is strongest for PFOA and PFOS other perfluorinated compounds could possess similar properties and the effects of mixed exposures constitute a potential risk which should not be underestimated.

While inclusions of immune related endpoints in OECD guidelines is an important step forward in protection of both the mature and developing immune system of wildlife and humans there still seem to be a need for tests that adequately test for dysregulated immune responses and late-life effects (Dietert *et al.*, 2007; IPCS, 2012). Future research should address these needs for knowledge of both potential effects on the immune system by environmental chemicals, the similarities and differences between species, sexes and in particular characterization of the developing immune system including the role and importance of the complex network of cells and their communication with each other, with immune organs and the rest of the organism.

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6 Discussion

This chapter contains a discussion of perfluorinated compounds and the immune system (6.1), thyroid hormone disruption (6.2), thyroid hormone disruption by environmental chemicals and model compounds (6.3) and protection of human brain development (6.4).

6.1 Perfluorinated compounds and the developing immune system

In this project, acute and developmental immunotoxicity induced by PFHxS was investigated (chapter 5). Surprisingly, almost no effects on functional immune endpoints were observed. However, the animal models needs optimization, as the positive control study also resulted in no convincing effects, leaving the study partially void. At the highest tested dose, PFHxS may reduce immune organ weights, but this could not be replicated in the main study that investigated lower doses. Hence, further studies of immunotoxicity of PFHxS are warranted.

Despite the limited effects in this project, both human and animal studies have previously shown that perfluorinated compounds can induce immunotoxic effects (section 2.6). In humans, effects on vaccine serum antibodies are convincing, whereas effects on other endpoints such as asthma or susceptibility to infections are more equivocal, especially with regards to PFHxS. Still, despite a limited number of studies, they suggest that current human exposure levels affect the immune system of the general population.
The fact that MMR vaccinations appear to modulate the effects of PFAS for asthma [Timmermann et al., 2017] indicate that the compounds have complex effect activities, not only on vaccine antibodies, but also in that they may cause a skewing of the immune system [G.-H. Dong et al., 2011, 2013; Zhu et al., 2016]. Hence, future research should focus on understanding the underlying mechanisms of PFAS mediated immunotoxicity and the potential implications for both the developing immune system and its long-term function. The combined effects of chemical exposures and other events impacting the immune system, for instance vaccinations and infections, should also be accounted for. In light of PFAS effects the endocrine systems also potential crosstalk-effects between the endocrine and immune systems should be investigated [WHO/UNEP, 2013]. Finally, bearing in mind the potential high human sensitivity to PFAS-mediated immune effects, *in vitro* tests using primary human immune cells could prove a beneficial addition to rodent models in understanding the molecular mechanisms driving the effects on the functional immune endpoints.

6.2 Perfluorinated compounds and thyroid hormone disruption

The effects of PFHxS on the thyroid hormone system (chapter 3 and 4) resemble effects observed for other perfluorinated compounds that consistently affect circulating levels of thyroid hormones in animals. Evidence from human studies is less consistent (section 2.5); however, the importance of thyroid hormone levels for brain development and the limitations of the epidemiological studies on thyroid function, in connection with ubiquitous exposure to PFAS warrants further investigations of the effects of PFAS exposure on the thyroid hormone system.

We observed a reduction in T4 following PFHxS exposure, but cannot deduce how much of the effect derives from TTR-binding or enzyme induction. This is in general a challenge for thyroid hormone disruption caused by increased hormone metabolism, as our understanding of causality and quantitative relationships still is very limited. Thus, future research should aim to determine what exactly the effects are when a chemical binds to TTR. In particular, determine how much T4 is displaced by a compound with a certain affinity and where this unbound T4 is subsequently dispositioned. In this context the liver response is especially relevant, as it remains unclear what the short- or long-term responses to unbound T4 are and to what degree T4 in itself can induce enzyme induction compared to what is induced by chemical exposure itself [Haberkorn et al., 2003]. Likewise, further studies of enzyme induction by compounds that do not affect distributor proteins are needed, as interpretations of results are currently impaired by the use of chemicals with multiple targets and differential effects. Through such studies, an AOP for increased metabolism of thyroid hormones could be further developed [Friedman et al., 2017] and understanding of the reasons and adverse effect implications for differential HPT axis activation after thyroid hormone disruption could be improved.

6.3 Thyroid hormone disruption by environmental chemicals and model compounds

Both drugs and environmental chemicals can affect thyroid homeostasis by acting on different different molecular targets. We are now characterizing which environmental chemicals might be thyroid hormone disruptors and if so, their potential downstream effects. And it is becoming increasingly clear that we need to adjust our assumptions, animal models and assays to fit a reality in which the environmental chemicals do not affect the thyroid hormone system similarly to the model compounds that shaped our understanding of the system in the first place.

To exemplify, the following section will discuss in more detail PFHxS- and PTU-induced thyroid hormone disruption, its downstream consequences and our ability to detect the adverse effects in a regulatory context. Finally, ending with recommendations for some of the considerations needed when evaluating thyroid hormone disruptors.

6.3.1 Contrasting downstream effects of the model compound propylthiouracil (PTU) and PFHxS mediated thyroid hormone disruption

As most of our knowledge of the thyroid system is derived from PTU studies, our studies on PFHxS were tailored for comparison with two PTU rat studies. One study from our own research group on behavioral effects [Axelstad et al., 2008] and another study from a collaborator [O'Shaughnessy, Wood, et al., 2018] on cortical gene expression (data from the study has also been published in [Gilbert et al., 2014, 2016, 2017; Johnstone et al., 2013; Wood et al., 2014]. Axelstad et al., (2008) used a study design similar to our PFHxS study with oral gavage exposure, while O'Shaughnessy et al., (2018) administered PTU in the drinking water from GD 6 at doses of 1, 2, 3 and 10 ppm PTU [Gilbert et al., 2014; Johnstone et al., 2013]. Some of the results are summarized in Table 2.

Table 2. Thyroid hormone profiles and effects after developmental exposure to PFHxS and PTU in similar studies.

Exposure, grouped according to effect on pup serum T4[a]	**T4, % of control**	**T3 % of control**	**HPT axis activation**	**Reference**
app. 50% of control				
25 mg/kg PFHxS[b]	55*	84*	↔	Ramhøj et al., 2018 & Ramhøj et al., manuscript
1 ppm PTU	54*	112	234%* ↑TSH	Johnstone et al., 2013
0.8 mg/kg PTU	50*	-	↑gland weight*[c]	Axelstad et al., 2008
20-40% of control				
2 ppm PTU	35*	108	448%*↑TSH	Johnstone et al., 2013
1.6 mg/PTU	23*	-	↑gland weight*[c]	Axelstad et al., 2008
0-20% of control				
3 ppm PTU	18*	82*	660%*↑TSH	Johnstone et al., 2013
10 ppm PTU[d]	8*	40*	392%*↑TSH	Johnstone et al., 2013
2.4 mg/kg PTU	16*	-	↑gland weight*[c]	Axelstad et al., 2008

*Indicates a statistically significant effect according to the paper.

[a]Pup serum T4 assessed on PN 14 in [O'Shaughnessy, Wood, et al., 2018]/[Johnstone et al., 2013] and PND 16 in other studies.

[b]No further effects on serum hormone levels in dams or offspring expected up to 45 mg/kg, according to range finding study, Ramhøj et al., 2018.

[c]TSH not measured.

[d]Dosing regimen described as "moderate" in the publication

Thyroid hormone profiles

PTU [Johnstone et al., 2013] and PFHxS caused differential serum hormone profiles (table 2 and chapter 4). While 25 mg/kg PFHxS and 1 ppm PTU both decreased T4 to 55% of control, T3 was decreased by PFHxS and increased by PTU. Additionally, PTU caused an increase in TSH while there were no effects by PFHxS. For PTU a dose of 3 ppm was. required to reduce T3. However, 3 ppm had also reduced T4 to 18% of control levels and increased TSH to 660%. Possibly, some of the differences were due to activation of the HPT axis caused by PTU; serum TSH goes up to maintain serum T3 levels, but at the

higher doses the compensation cannot maintain T3. Hence there are marked differences in the effects on serum hormones achieved in the O'Shaughnessy et al., PTU study and the PFHxS study, which could indicate differences in downstream effects of the compounds.

Cortical gene expression

The primary function of thyroid hormones is regulation of gene transcription. Thus, transcription of TH regulated genes could be a downstream effect of thyroid status in specific tissues at specific times [Bernal, 2007, 2017; Crofton et al., 2018; Royland et al., 2008]. In other words, cortical gene transcription should be affected if low serum T4 correlates with low cortical T4, as is the case in some models [Gilbert et al., 2013; Morreale de Escobar et al., 1988; Porterfield & Hendrich, 1993; Ruiz de Oña et al., 1988; Schwartz et al., 1997].

O'Shaughnessy et al., showed dose-response relationships between cortical gene transcription at PN 14 and reduced serum T4 after developmental exposure to PTU in drinking water (table 2) [O'Shaughnessy, Wood, et al., 2018]. Seven genes were significantly affected at 1 ppm PTU where brain T4, but not T3, was affected. Gene transcripts were decreased with decreasing levels of T4, down to below 0.1 fold of control expression, providing a compelling case for their transcriptional regulation being influenced by thyroid hormones [Bastian et al., 2012; Bernal, 2017; Gil-Ibañez et al., 2017; O'Shaughnessy, Wood, et al., 2018; Royland et al., 2008]. Unfortunately, at the low dose of 1 ppm PTU (T4 at 54% of control) a reduction of only 0.3 fold is observed, arguably falling within the inherent variability of the assay. Hence, reductions of this magnitude, despite statistical significance, are only confirmed by the remainder of the dose-response relationship. Extrapolating this observation to studies of environmental chemicals, which might only produce reductions of about 50% in circulating T4 in the pups on PN 14, it would result in an expression level at 0.7 fold compared to the control – in most cases not enough to be deemed significant and reliable. This is exactly what was observed in the PFHxS study where some of the same genes were investigated (chapter 4). The submaximal T4 reduction by PFHxS is not the more severe hypothyroxinemia that would be necessary to verify whether transcription of the thyroid sensitive genes examined by O'Shaughnessy et al. were affected.

Cortical gene transcription as a downstream endpoint could possibly be enhanced by dosing pups directly with the thyroid hormone disruptor to induce a severe hypothyroxinemia for better comparison with PTU studies. This would exploit the nature of the direct relationship between concurrent TH levels and downstream gene transcription in the postnatal pups. And simultaneously be in contrast to other downstream effects which are assessed later in life and represents readouts of thyroid function over time (fig. 6).

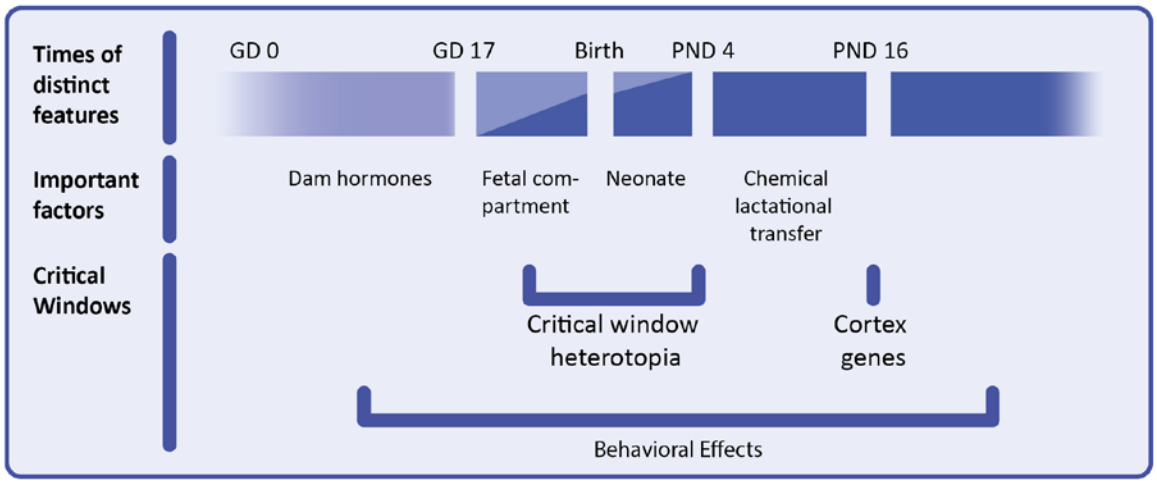

Figure 6 | Conditions of thyroid hormones and downstream endpoints during development. Chemical interference with thyroid hormones should be assessed in light of the distinct features characterizing the developmental periods of the rat. In the beginning of gestation dam thyroid hormone balance is determining for the fetal supply, later follows gradual onset of fetal thyroid function and thyroid hormone balance in both maternal and fetal compartments have an influence on fetal TH supply. Birth denotes a sharp transition to new compartmental conditions with rapid changes in neonate HPT axis and hormones. In the postnatal period disruption of pup thyroid hormones can only take place with sufficient lactational transfer of the chemical. After PND 16 dosing through the drinking water can be efficient as direct exposure route. Hence, many factors determine the induction of severe hypothyroxinemia during brain development and the presence of hypothyroxinemia during the critical window of an effect. The outcomes of downstream endpoints are determined by thyroid hormone balance during the endpoint-specific critical windows, e.g. Heterotopia formation is dependent upon T4 deficiency from GD 20 to approximately PND 4, a critical window which spans two distinct developmental conditions and which is compromised with adequate T4 supply during one of them. GD: Gestational day, PND: Postnatal day.

Behavior, learning and memory

Effects on behavior, including learning and memory, represent functional defects caused by impaired brain development and assessment can take place later in life, long after exposure cessation. Accordingly, the relationship with brain hormones may be more complex, as effects may depend on a certain TH deficit during a particular developmental period; but perhaps more often during the entire period of brain development (fig. 7) [Crofton, 2000; O'Shaughnessy, Kosian, et al., 2018; Zoeller et al., 2007; Zoeller & Rovet, 2004]. However, Axelstad et al., (2008) showed reasonable correlations between PTU-induced decreased pup (PND 16) and dam (GD 15) hormones and effects on brain development. These effects were observed in the radial arm maze and on motor activity levels after T4 reductions to 23 and 16% of control on PND 16, respectively, and there were no effects at T4 at 50% (Table 2) [Axelstad et al., 2008]. Therefore, the sensitivity of

the employed behavioral assays to detect developmental thyroid hormone disruption is limited to very severe T4 reductions and it is not surprising that the high dose PFHxS (T4 at 55% of control) did not result in TH mediated effects (chapter 4).

6.3.2 Sensitivity of assays for downstream effects of thyroid hormone disruption

Adverse effects on human brain development after developmental hypothyroxinemia typically manifest with complex functional parameters such as IQ and language [Berbel et al., 2009; Gyllenberg et al., 2016; Haddow et al., 1999; Henrichs et al., 2010; Korevaar et al., 2016; Román et al., 2013]. Yet, the testing of chemicals for effects on the developing nervous system relies on rather simple behavioral tests in rodents [Hass, 2006]. We are thus faced with two primary problems. Firstly, there is a discrepancy between the complexities of the human brain and rodent brain, and consequently parameters that can be evaluated in rodents. Adverse effects in rodents are typically only detected at severely reduced T4 levels, whereas smaller reductions in T4 may cause adverse effects in humans. To the former, studies have shown similar picture of limited adverse effects correlated to submaximal thyroid hormone reduction after chemical exposure [Axelstad, Boberg, Hougaard, et al., 2011; Axelstad, Boberg, Nellemann, et al., 2011; Crofton, 2004; Gilbert et al., 2013; Gilbert & Lasley, 2013; Gilbert & Sui, 2008; Harry et al., 2014; Kodavanti et al., 2010; Kortenkamp et al., 2017; Noda et al., 2005]. Unfortunately, in animal studies of PFHxS and other environmental chemicals there is a limit to the maximal obtainable effect on T4 levels during the specific developmental stages: lower chemical potency, limited chemical transfer to pups or excessive pup or maternal toxicity at higher doses not related to thyroid effects, all limit the T4 reductions [Axelstad et al., 2013; Axelstad, Boberg, Hougaard, et al., 2011; Axelstad, Boberg, Nellemann, et al., 2011; Crofton et al., 2000, 2005; Kodavanti et al., 2010]. Hence, due to assay insensitivities it is often not possible to distinguish between absence of effect, no effect due the existence of adequate brain hormones, and no effects as a result of only mild TH reductions in the fetal or pup compartments. Overall, after submaximal thyroid hormone disruption in animal studies, we have no reliable downstream endpoint in the brain. At reductions of 0-50% in circulating thyroid hormones there is virtually no potential candidates for such a reliable endpoint (fig. 7). Some genes, and potentially periventricular heterotopias may be affected around a 50%-ca.75% reduction in the offspring and the fetus, respectively (see below). This therefore leaves only a putative threshold at about 50% for detection of adverse

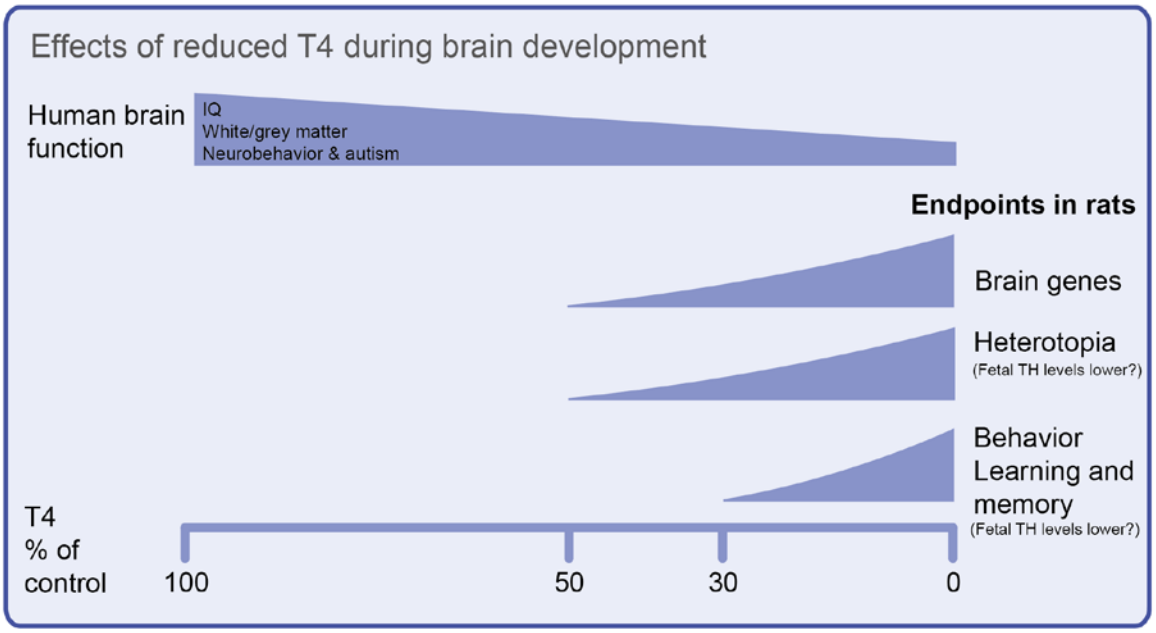

Figure 7 | Schematic correlations between decreased T4 levels during development and effects in humans and rats. While effects on functional brain endpoints appear in humans when the maternal fT4 levels are in the lower end of the population reference interval, current endpoints in rats are only affected after T4 levels at less than 50% of control levels as assessed postnatally in the pups. Note that potentially both heterotopia and behavior may be dependent on thyroid hormone insufficiency during critical windows earlier in development. IQ: Intelligence quotient TH: Thyroid hormones.

effects on rat brain development after thyroid hormone disruption. This prominent lack of endpoints suitable for regulatory evaluation of thyroid hormone disruption presents a serious challenge for testing of thyroid hormone disrupting chemicals and thereby the protection of human health.

Currently, the best candidate for an adverse effect suitable for large scale toxicological studies may be periventricular heterotopia, a structural effect (adverse effect in terms of toxicological regulation) observable at around 50% decrease in T4 relative to control at PN14, [Gilbert et al., 2014; Goodman & Gilbert, 2007; Hassan et al., 2017]. Heterotopia seems to be dependent upon a small critical window around birth, however. Late during gestation, a dose of 1 ppm PTU decreases fetal T4 to around 40% of control levels and the heterotopia was just detected [Gilbert et al., 2014; Spring et al., 2016]. So far, heterotopia

has been observed after exposure to methimazole, PTU, and perchlorate, all sharing an intrathyroidal mode of action [Berbel et al., 2001; Gilbert et al., 2014, 2018; Goodman & Gilbert, 2007; Powell et al., 2012; Shibutani et al., 2009; Shiraki et al., 2012; Spring et al., 2016]. Further studies are needed to assess both the exact sensitivity of the assay and the presence of effects after thyroid hormone disruption through extrathyroidal modes of action.

6.3.3 Considerations for testing of environmental chemicals in contrast to model compounds

Our knowledge of the thyroid system has relied heavily on animal models of severe hypothyroidism. As data on environmental chemicals is now emerging, a number of outstanding challenges remain to be addressed.

- First, as described, development of a reliable endpoint that can be applied within the framework of toxicological studies, e.g. not scanning of the brain or very specific counting of cell migration. Of special interest is an endpoint sensitive to decreases in T4 of less than 50%.

- Second, model compounds and thyroid hormone disruptors affect TH serum hormone profiles in dams, fetus and offspring differently. Knowledge of the kinetics over time in different compartments and potential compensatory mechanisms will be crucial for interpretation of the significance of the presence/absence of an adverse effect after chemical exposure.

- Third, characterization of how different modes and mechanisms of perturbing the thyroid system affect circulatory TH and the peripheral tissue TH status, especially brain thyroid hormone concentrations and the derived effects.

- Fourth, knowledge about local peripheral compensatory mechanisms is lacking. For example, an understanding of the compensatory capacity of D2 induction in the brain and its implications for adverse effects would be relevant.

- Fifth, one specific and puzzling finding remains; the source of the lack of a compensatory TSH increase in response to thyroid hormone disruption by some environmental chemicals. The source(s) and downstream consequences of this type(s) of thyroid hormone disruption are still elusive.

6.4 Protection of human brain development

The challenge of safeguarding human brain development from exposure to xenobiotic chemicals disrupting thyroid hormone system is complex. Currently, it relies on the development of tests and endpoints for thyroid hormone mediated adverse effects. As this approach has limitations and may be prolonged, this section will discuss more recent epidemiological data indicating the need for an alternative and timely approach to ensure optimal human brain development of future generations.

6.4.1 Defining a threshold for adverse effect of developmental hypothyroxinemia in humans

Thyroid hormones are necessary for human brain development, yet we do not know the lower (or upper) threshold for an adverse, suboptimal brain development due to developmental thyroid hormone insufficiency. It seems that the variation within populations and analytical methods (section 2.4.3) impedes the setting of an absolute limit for thyroid hormone sufficiency. Furthermore, we are lacking knowledge of the consequences of a dynamic T4 reduction in an individual as opposed to the effects that are linked to having a low fT4 within the reference interval. However, what can be derived from the epidemiological studies is knowledge about the impact on brain development of even very small differences in pregnancy T4 levels.

Per definition, studies of developmental hypothyroxinemia deals with pregnant women that have T4 levels within the reference interval of the population, often studying differences between the lowest 5^{th} and/or 10^{th} percentile and the rest of the population (section 2.4.2). In Berbel et al., (2009) the lowest 10^{th} percentile had an upper fT4 limit 15.4% above the lower limit of the reference interval. Thus effects on neurodevelopment were found for children of mothers within this 100-115.4% T4 (compared to lower reference boundary) [Berbel et al., 2009]. Another study found divergent results between mild and severe hypothyroxinemia compared to the rest of the population. Hence a reduction of 6.8% of fT4 from the upper limit had significance for the outcome, as expressive language was affected in both groups but only severe maternal hypothyroxinemia predicted a higher risk of nonverbal cognitive delay [Henrichs et al., 2010]. These studies have indicated the importance of very small differences in fT4 during gestation. Further supporting this

notion is an inverted U-shaped curve for IQ as a function of fT4, and also a clear dose response relationship between the lowest ca. 10^{th} percentile of fT4 levels and IQ [Korevaar et al., 2016]. This dose response relationship suggests that even small decrements in the availability and function of TH have an effect on human brain development and the effect has proven clinically relevant in other studies. Similar dose response relationships have been seen for neurobehavioral development [Gyllenberg et al., 2016; Pop et al., 2003].

It is thus evident that, although the exact threshold for adverse effect on neurobehavioral development is yet unknown, very small differences in fT4 levels has an impact on brain development. At least in the lower end of the reference interval this impact takes place in a dose-response like manner, potentially with a threshold effect for the start of the dose response relationship. Absolute T4 levels for both threshold and dose-response curve are probably dependent on both the population investigated and analytical method at hand and they are influenced by other factors as iodine status and TPO-ab+ (section 2.4.3). If environmental chemicals also have an impact on TH of populations they may cause a shift of the population normal distribution and transfer more people into the dose-response area for neurobehavioral effects.
Despite these unknowns, our current knowledge shows that human brain development is sensitive to thyroid hormone disruption also in the range below 10% changes in fT4. This indicates the need for an alternative approach in the evaluation of thyroid hormone disrupting chemicals.

6.4.2 T4 as a marker and adverse effect outcome in *in vivo* studies

Presently, a sensitive assay to detect adverse effects caused by developmental T4 insufficiency is lacking. With the recognized importance of fT4 to human development and health, there is a strong need to change how we currently asses chemicals for thyroid disruption. Therefore, based on current knowledge, I would propose directly using serum T4 levels as adverse effect outcome instead of downstream effect markers.

Arguments for adopting serum T4 levels as an adverse effect outcome for risk assessment purposes are:

- Serum T4 levels are currently used as marker of thyroid function both in humans and in OECD guideline studies on rats.

- In human epidemiological studies, it is small changes in serum T4 and not T3 nor TSH that display strongest correlation with effects on child neurobehavior.

- Assessment of serum T4 is an established clinical method to diagnose and manage thyroid disorders with obvious advantages; e.g. ease of routine sampling and automated analyses.

- Serum T4 levels are directly translatable between humans and rats when used as a marker of thyroid function. The potential sensitivity of the rat thyroid system can be taken advantage of to account for the sensitivity of human brain development.

There are, however, some shortcomings with respect to serum T4 levels as a biomarker that must be considered. They include:

- Measurements of circulating T4 levels do not necessarily reflect changes at the local tissue levels, where T4 exert its functions.

- In developmental toxicity studies, T4 levels are measured in dams and not in the fetal compartment. Although dam serum hormones are very important for fetal brain development, fetal thyroid hormones can be more sensitive than dam thyroid hormones.

- Chemicals with MIEs in peripheral tissues, e.g. inhibition of deiodinases, membrane transporters or interference with thyroid hormone receptors, may not be detected by assessment of serum T4 levels.

Despite these limitations, serum T4 is presently the best option for assessing thyroid hormone status in animal toxicity studies, not least since even slight effects on circulating T4 level has been shown to have serious effects on human brain development. But efforts should still be made at developing even better and more sensitive test methods for thyroid disruption, as a golden standard test, or test battery, remains needed. To account for the potential insensitivity of T4 levels as adverse outcome readout, safety factors should, for the time being, be applied to account for potential specific effects in the sensitive fetal compartment and for peripheral targets of thyroid disrupting chemicals.

7 Conclusions

This chapter contains the conclusions on the work presented in this thesis

Conclusions

The overall aim of this PhD project was to better define to what degree early-life exposure to perfluorinated chemicals can affect thyroid hormone signaling and in extension, brain development. A sub-aim was also to examine potential effects on the immune system. To achieve this, we exposed pregnant rats to PFHxS and examined appropriate effect end-points in a series of experiments.

The presented developmental toxicity studies have extended our knowledge on how perfluorinated chemicals, in this case PFHxS, can disrupt normal development. We saw clear signs of effects in the liver, but also indications that PFHxS, when combined with a background exposure to endocrine disrupting chemicals, can disturb fetal hormone signaling and thereby affect sexual differentiation of the brain.

This thesis also shows how PFHxS exposure can reduce circulating T4 and T3 in both dams and offspring without a compensatory increase in TSH levels. And, although we could not correlate developmental thyroid hormone insufficiency to effects in our behavioral tests, comparisons to PTU studies show that this is not unexpected since the magnitude of changes to thyroid hormone levels induced by PFHxS were moderate compared to the maximal effect sizes induced by PTU. The effects on circulating thyroid hormone levels were nevertheless of a magnitude that raises concern for human brain development, as changes of just a few percent in maternal fT4 levels are associated with impaired cognitive and neurobehavioral development in their children; but also because current human exposures include other thyroid hormone disrupting compounds.

Our understanding of the thyroid system and compensatory mechanisms is improving, but when it comes to effects caused by xenobiotics, our knowledge is still limited. Worryingly, the presented work has highlighted that current behavioral assays are not sensitive enough to detect adverse effects of developmental thyroid hormone disruption. It is critical that we become capable of detecting adverse effects caused by less than a 50% reduction in thyroid hormone levels. It is well known that even small reductions in available thyroid hormones in humans can have negative consequences for the children's brain development. Then, as environmental chemicals can cause T4 reductions, there is a strong need for implementing serum T4 levels as an adverse outcome in itself. A suggestion for effect size could be statistically significant effects at a magnitude of 10-20% from control levels.

Due to technical difficulties, we were unable to establish a clear link between PFHxS exposure and immunotoxicity. However, available literature on PFHxS and other PFASs

suggests that they are immunotoxic in humans at current exposure levels. Therefore, it is imperative that this work continues and that we establish with more certainty what the long-term consequences of PFAS exposure to human health may be.

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