In vitro and *in vivo* interactions of organohalogens with the endocrine system – the role of metabolites and implications for human health

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Proefschrift

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Stellingen

1. Maternale blootstelling aan gehydroxyleerde PCB-metabolieten veroorzaakt bij nakomelingen van drachtige ratten subtiele gedragsveranderingen en versnelt de reproduktieve veroudering bij concentraties die slechts een orde van grootte boven de huidige PCB-metaboliet concentraties in humaan navelstrengbloed liggen (*dit proefschrift*).

2. De produktie en het gebruik in een veelheid aan toepassingen van polygebromeerde difenyl ethers zou, gezien hun persistentie, bioaccumulatie en toxische effecten waaronder de verstoring van de schildklierhormoon-huishouding, zo snel mogelijk verboden moeten worden (*dit proefschrift; The Swedish National Chemicals Inspectorate, 15 March 1999).*

3. De binding van stoffen aan TTR *in vitro* leidt niet per definitie tot een verstoring van de schildklierhormoon-huishouding *in vivo* (*dit proefschrift*).

4. Bij het onderzoek naar de mogelijke endocriene verstoring van stoffen wordt de schildklierhormoon-achtige werking van stoffen geheel onderschat.

5. Het voorstel van de Environmental Protection Agency (EPA) om vele chemicaliën te testen op endocriene verstoring *in vivo* is noodzakelijk, maar is in strijd met hun eigen beleid ter beperking van het aantal dierproeven.

6. De *in vivo* Micronucleus test zou voor registratie van stoffen niet gebruikt mogen worden indien niet duidelijk is of de betreffende stof het beenmerg bereikt.

7. De IC_{50} concentratie (de concentratie waarbij 50% inhibitie optreedt) van een stof voor de remming van een cytochroom P450 enzym bij één substraatconcentratie geeft geen enkele extra informatie als de wijze van remming niet bekend is.

8. Niets zal een grotere positieve invloed hebben op de gezondheid van de mens en de overlevingskansen van het leven op aarde dan de evolutie naar een vegetarisch dieet (*A. Einstein*, 1879-1955).

Stellingen, behorende bij het proefschrift *"In vitro* and *in vivo* interactions of organohalogens with the endocrine system – the role of metabolites and implications for human health" van Ilonka A.T.M. Meerts, te verdedigen op vrijdag 2 november 2001.

Learn from yesterday,

live for today,

hope for tomorrow.

The important thing is to not stop questioning.

Albert Einstein (1879-1955)

Contents

CHAPTER 1

General introduction

Preface

The research described in this thesis was part of an international study funded by the European Community, focussed on the **R**isk of **EN**docrine **CO**ntaminants on human health. The **RENCO** study was initiated at a time when an increasing number of papers showed that several classes of environmental contaminants were able to induce endocrine activity *in vitro* and *in vivo*. The detection of some of these endocrine active environmental contaminants (such as hydroxylated polychlorinated biphenyls, OH-PCBs) in human blood raised concern about the possible consequences for human health, especially because *in vivo* studies with pregnant rats and mice had shown that hydroxylated PCBs were able to accumulate in their fetuses. The RENCO study was aimed at investigating the possible human risks of background environmental exposure to endocrine active organohalogen compounds. Special emphasis was placed on hydroxylated polychlorinated biphenyls (PCBs) and related phenolic organohalogens with a high fetal accumulation potential. The work in this thesis presents the investigation of the toxic (endocrine mediated) potencies of "new" environmental contaminants, which were detected in human blood as part of the RENCO study. In the first part of the research the *in vitro* endocrine potency of selected brominated and chlorinated compounds was investigated. In the second part of the research, *in vivo* studies with an endocrine active model compound were performed in order to compare *in vitro* and *in vivo* endocrine activity and to predict the possible adverse effects of these compounds on human health.

Organohalogen compounds – environmental occurrence

During the last half century, vast quantities of diverse synthetic chemicals have entered the environment because of their extensive use in agricultural, industrial or household applications. These chemicals include herbicides, insecticides, fungicides, and industrial chemicals, like polychlorinated biphenyls (PCBs), and breakdown products of detergents (like e.g. nonylphenols). The source of entry in the environment is determined by the intended use of the compound. Herbicides, insecticides and fungicides are intentionally released into the environment at the point of application. PCBs and penta- to nonylphenols are an example of unintentionally released compounds, which enter the environment by volatilisation, leakage or leaching either during a product's lifetime or after disposal.

In addition, some chemicals (e.g. dioxins) are unintentionally produced as by-products of industrial or combustion processes. Dependent on their structure and the physico-chemical properties, compounds can be widely distributed and can bioaccumulate in the environment. The most persistent compounds are organohalogen compounds which possess a low water solubility and a high lipid solubility, that resist chemical and biological degradation, and are able to bioaccumulate in fatty tissues of living organisms. The contaminants determined in wildlife and humans in the highest concentration at the moment are polychlorinated biphenyls (PCBs), 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) and hexachlorobenzene (see Figure 1.1). These organohalogen compounds belong to the class of persistent organic pollutants (POPs), meaning that they possess toxic characteristics, are persistent, are liable to bioaccumulate, are prone to long-range atmospheric transport and deposition, and can result in adverse environmental and human health effects at locations near and far from their sources (UN-ECE, 1998).

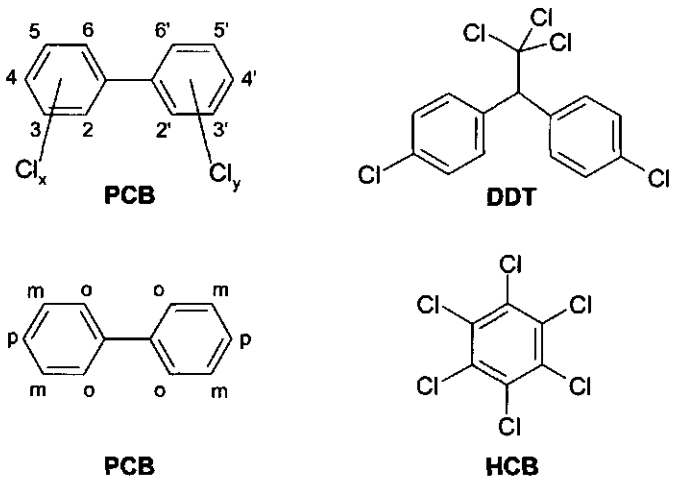

Figure 1.1. Structures of several persistent organic pollutants. PCB: polychlorinated biphenyl, DDT: 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane, HCB: hexachlorobenzene (HCB), o: ortho, m: meta, p: para.

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) – "old" versus "new" environmental contaminants

Polychlorinated biphenyls (PCBs, Figure 1.1) are a group of chlorinated hydrocarbons consisting of 209 possible congeners ranging from three monochlorinated isomers to the fully chlorinated decachlorobiphenyl isomer. Their physico-chemical properties are dependent on the degree of chlorination. Generally, PCBs with a high degree of chlorination have a low water solubility and high lipophilicity. PCBs were produced in large quantities as mixtures varying in the degree of chlorination. Their major use was as dielectric fluids in transformers and capacitors. Some of their applications resulted in direct or indirect releases into the environment and large amounts were

released due to inappropriate disposal, accidents and leaks from industrial facilities. PCBs have been identified in environmental samples as early as the 1960s (Jensen, 1966; Jensen, 1972; Koeman *et al.*, 1969, 1972, 1973) and can be detected in almost all compartments of the biosphere at the moment. Concentrations are particularly high in marine wildlife species at high trophic levels, such as in fish-eating birds (Helander *et al.*, 1982; Olafsdottir *et al.*, 2001), seals (Addison and Stobo, 2001; Cleemann *et al.*, 2000; Watanabe *et al.*, 1999), whales (Minh *et al.*, 2000) and polar bears (Letcher *et al.*, 1995; Norstrom *et al.*, 1998; Sandau, 2000). Even human milk samples and adipose tissue contain PCBs and other organohalogen compounds (Loganathan and Kannan, 1994; Norén, 1993; Schade and Heinzow, 1998).

Because of the ban on the production and use of PCBs in the early 1980s, time trend studies now show a decline in PCB (and also DDT) concentrations in e.g. freshwater fish in the United States (Schmitt *et al.*, 1999), in tissues of various biota (fish, seals, birds) of the Baltic Sea (Olsson *et al.*, 1997) and in mother's milk (Norén, 1993; Norén and Lundén, 1991; Schade and Heinzow, 1998). However, leakage of PCBs from old equipment, building materials, stockpiles and landfill sites constitutes a continued threat of PCB emission. Indeed, some time trend studies of PCB concentrations in human adipose tissue show no significant decline, thus indicating that humans are still continuously exposed to PCBs (Loganathan and Kannan, 1994).

Unfortunately, PCBs are not the only environmental pollutants posing a threat to humans and wildlife. In addition to the organohalogen compounds which are on the list of persistent organic pollutants, several other organohalogens of which we know much less about are produced in high quantities at the moment. Over the last 10 to 15 years, increasing concentrations of a new class of organohalogen compounds, the polybrominated diphenyl ethers (PBDEs), have been measured in the environment and human tissues. PBDEs are used as additive flame retardants, at concentrations of 5-30% in many different polymers, resins and substrates for applications in electronic devices, circuit boards in personal computers and television sets, building materials and textiles (Pijnenburg *et al.*, 1995; IPCS, 1994). The reason for using brominated compounds as flame retardants is based on the ability of halogen atoms, which will be released by thermal decomposition of the bromo-organic compound, to chemically reduce and retard the development of fire. The annual world production of flame retardants is roughly 600,000 metric tons, of which about 60,000 tons are chlorinated and 150,000 tons are brominated compounds. Of the brominated flame retardants, about one third contain various brominated compounds (including polybrominated biphenyls), another third contain tetrabromobisphenol A and derivatives, and the last third contain PBDEs (OECD, 1994).

The molecular structure of PBDEs is quite comparable to PCBs (Figure 1.2), however, as a result of the ether bridge between the two phenyl rings PBDEs are not able to adopt a planar conformation, regardless of the substitution pattern of the bromines. The theoretical number of possible PBDE congeners is also 209; the different congeners are divided into 10 groups (from mono- to decabromodiphenyl ethers). Commercial mixtures of PBDEs contain an average bromine content substitution number of 5 (pentabromodiphenyl ether, pentaBDE), 8 (octabromodiphenyl ether, octaBDE) or 10 (decabromodiphenyl ether, decaBDE). The technical decaBDEs have the widest industrial use. The number of different congeners in each PBDE mixture is quite small compared to the number of PCB congeners commonly found in mixtures or in the environment (IPCS, 1994). This

is probably because the reaction used to synthesise brominated diphenyl ethers (i.e. direct bromination of diphenyl ether in the presence of a catalyst; IPCS, 1994) is fairly selective (Sjödin *et al.*, 2000).

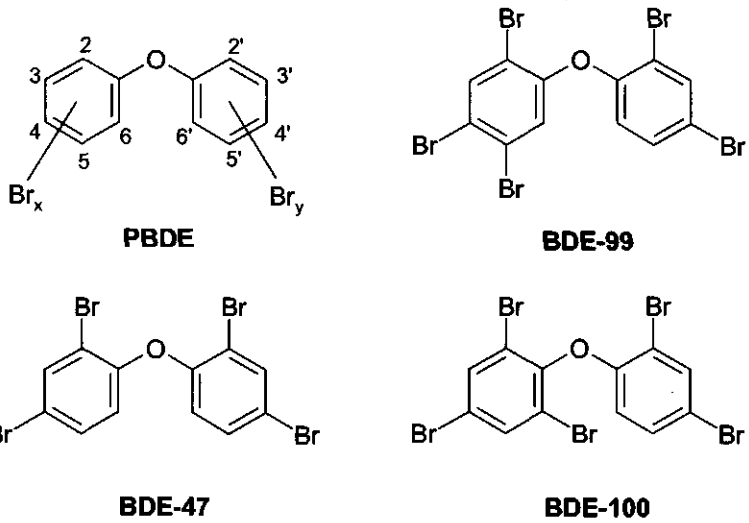

Figure 1.2. Structure and ring positions of PBDEs, and structure of the most common PBDEs detected in the environment. BDE-47: 2,2',4,4'-tetraBDE; BDE-99: 2,2',4,4',5-pentaBDE; BDE-100: 2,2',4,4',6-pentaBDE.

PBDEs are lipophilic and their solubility in water is low. Commercial PBDEs are quite resistant to physical, chemical and biological degradation (IPCS, 1994). Because of their physico-chemical properties and the application of PBDEs as additive flame retardants, which can leach and escape from the existing polymers much more easily than e.g. reactive flame retardants (IPCS, 1997), it is not surprising that PBDEs have been detected in the environment and in wildlife species. PBDEs have been identified in various biotic samples, such as fish-eating birds and marine mammals (Jansson *et al.*, 1987), shellfish and sediment (Haglund *et al.*, 1997), and also in human blood (Sjödin *et al.*, 1999, 2000) and breast milk (Meironyté *et al.*, 1999). So far, the total PBDE concentrations detected in wildlife and humans are lower than the concentrations of total PCBs. On a congener basis, the levels of individual PBDEs in these samples could be similar to those of individual PCB congeners. However, the levels of total PBDEs are lower because fewer congeners are present in technical mixtures and in the environment (Darnerud *et al.*, 2001). The most dominant congeners found in wildlife and humans are generally 2,2',4,4'-tetraBDE (BDE-47), 2,2',4,4',5-pentaBDE (BDE-99) and 2,2',4,4',6-pentaBDE (BDE-100)[1]. Although decaBDE is not commonly found in wildlife and humans, this does not imply that decaBDEs do not pose a threat to the environment.

[1] The numbering of PBDE congeners is adapted from the International Union of Pure and Applied Chemistry (IUPAC) numbering system originally designed for PCBs (Ballschmiter and Zell, 1980).

DecaBDE is poorly absorbed in organisms, but debromination of decaBDE occurs in ultraviolet light and sunlight, leading to a formation of lower brominated PBDEs and various brominated dibenzofurans (Watanabe and Satsukawa, 1987), which can then be absorbed by organisms or humans.

While the levels of PCBs in human blood have been shown to be stable or decreasing (as described earlier in this chapter), PBDE concentrations in human milk sampled in Sweden from 1972 to 1998 increased from 0.07 to 4.02 ng/g lipid weight (Meironyté *et al.*, 1999). Because PBDEs were shown to be widely distributed in the environment, the need for restrictions on certain PBDEs in different types of plastics and textiles is currently being discussed within the EU. The Organisation for Economic Co-operation (OECD) published a document with the recommendation to stop the use of mainly tetra- and pentaBDEs (OECD, 1994).

Hydroxylated organohalogen compounds

Although the metabolism of xenobiotics, including PCBs, generally results in the formation of more polar metabolites which are subsequently cleared from the organism, several hydroxylated metabolites of PCBs (OH-PCBs) have been identified in the blood of marine mammals, polar bears, fish-eating birds and humans (Bergman *et al.*, 1994; Klasson-Wehler *et al.*, 1998; Sandau *et al.*, 2000; Sjödin *et al.*, 2000). The amount of OH-PCBs detected in human blood is at concentrations of 10-30% of the parent PCB concentration (Sandau *et al.*, 2000, Sjödin *et al.*, 2000) but as high as 2-3 times the parent PCB level in Polar bear blood (Sandau, 2000). Normally, hydroxylated aromatic compounds are not bioaccumulated in lipids, unless they are bound to proteins. The OH-PCBs retained in human blood possess a common structural element, namely an OH-group in the *para*-position, or occasionally in the *meta*-position, with chlorine atoms on the adjacent carbon atoms, and at least one chlorine atom on the non-OH containing phenyl ring in the *para*-position (Letcher *et al.*, 2000). These structural elements are in accordance with the structural requirements for binding of an OH-PCB to human transthyretin (TTR), a transport protein in human blood which normally transports thyroxine (T_4), the natural thyroid hormone (Lans *et al.*, 1993). Figure 1.3 shows the resemblance of some OH-PCBs retained in human blood with T_4. The majority of OH-PCBs detected in human blood are formed from the more persistent PCB congeners with 5 to 7 chlorine atoms (Klasson-Wehler *et al.*, 1997; Sandau *et al.*, 2000, Letcher *et al.*, 2000).

Hydroxylated and methoxylated PBDEs (OH-PBDEs and MeO-PBDEs) have been detected in various biotic samples from the Baltic Sea (Asplund *et al.*, 1999; Haglund *et al.*, 1997). Concentrations of OH-PBDEs in blood plasma from Baltic salmon were estimated to be about 30-50 ng/g lipid weight, similar to concentrations of the major PBDEs in these samples (Asplund *et al.*, 1999), thus suggesting that also OH-PBDEs are selectively retained in blood plasma by binding to proteins. Apart from these anthropogenic sources of OH-PBDEs, certain marine sponges are able to form hydroxylated PBDEs (Carte and Faulkner, 1981; Fu *et al.*, 1995).

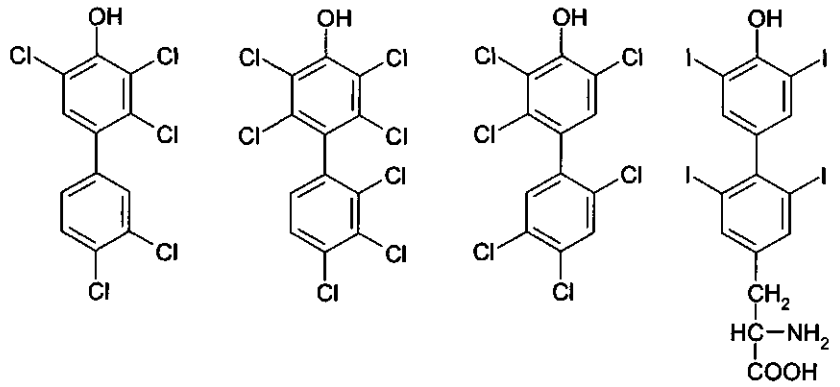

Figure 1.3. The resemblance of several hydroxylated PCBs determined in human blood with the thyroid hormone thyroxine (3,3',5,5'-tetraiodo-L-thyronine, T_4).

Organohalogen compounds – toxicity

PCBs and OH-PCBs

The toxic effects of PCBs have been extensively studied and reported. PCBs induce a broad range of toxic effects, including hepatic porphyria (Vos and Koeman, 1970), neurotoxicity, developmental toxicity, reproductive toxicity, teratogenicity and carcinogenesis (reviewed in Peterson *et al.*, 1993; Safe 1990, 1994; Schantz 1996; Seegal 1996; Tilson and Kodavanti, 1997). The toxicity of PCBs is dependent on their chlorine substitution pattern, and is often divided into dioxin-like toxicity (in case of planar and mono-*ortho* PCBs) and non-dioxin-like toxicity (poly-*ortho* PCBs). The most toxic PCBs are the dioxin-like PCBs, of which the mechanism of action is mediated by the aryl hydrocarbon receptor (AhR; Poland and Knutson, 1982). Dioxin-like compounds have been shown to bind to this AhR after entering cells (Safe, 1992). The AhR then undergoes a process of activation and moves to the nucleus, where it is bound to specific elements (the dioxin response elements, DRE) on the DNA. This results in increased transcription of genes that possess a DRE element in their upstream control for expression, such as cytochrome P450 1A1 and 1A2 genes. Since AhR binding affinity, enzyme induction and toxic potencies correlate well over a wide range of different congeners, it is nowadays widely accepted that the AhR plays a major role in the onset of toxicity of many organohalogen compounds. The non-planar PCB congeners do not bind to the AhR and have been shown to induce toxic effects such as tumour promotion (Silberhorn *et al.*, 1990; Van der Plas *et al.*, 2000), alterations in plasma thyroid hormone (McClain *et al.*, 1989; Barter and Klaassen, 1992, 1994) and neurotoxicity (Seegal *et al.*, 1990, 1996, 1997; Shain *et al.*, 1991). The mono-*ortho* PCB congeners have intermediate toxicity and bind to the AhR with less affinity than planar congeners.

During the last decades it has become evident that PCBs and especially OH-PCBs can directly interfere with the endocrine system, including the thyroid, retinoid, and estrogen system

(Brouwer and Van den Berg, 1986; Jansen *et al.*, 1993; reviewed by Brouwer *et al.*, 1998 and Brucker-Davis, 1998). The interactions of PCBs with these endocrine systems are described in more detail in the next paragraphs.

Interactions of PCBs and OH-PCBs with the thyroid hormone system

For OH-PCBs as well as parent PCBs and other organohalogen compounds (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, DDT, hexachlorobenzene and pentachlorophenol) it is well known that thyroid hormone levels are affected in experimental animals (Brouwer, 1989; reviewed in Brouwer *et al.*, 1998; Brucker-Davis, 1998). The interaction of (OH-)PCBs takes place at multiple levels of the thyroid hormone system, including the thyroid gland, the pituitary-thyroid feedback system, transport of thyroid hormones and enzymatic conversion of thyroid hormones. Decreased levels of circulating plasma thyroxine (T_4) following PCB exposure have been shown in both adult (Byrne *et al.*,1987; Barter and Klaassen, 1994; Van den Berg *et al.*, 1988) and developing organisms (Collins and Capen 1980; Darnerud *et al.*, 1996; Morse *et al.*, 1993, 1996a; Ness *et al.*, 1993; Seo *et al.* 1995). All categories of PCB congeners (planar, nonplanar and mono-*ortho* PCBs) are able to alter thyroid function, although through different mechanisms. The main mechanism of thyroidogenic effects of planar PCBs is thought to be mediated through the AhR, namely the induction of UDP-glucuronosyltransferases (UDP-GT). As a result of this induction, the biliary excretion of T_4 is enhanced, leading to decreased plasma levels of T_4 (Barter and Klaassen, 1992; Bastomsky, 1974; Van Birgelen *et al.*, 1995). Indeed, there was a good and statistically significant relationship between decreased plasma T_4 levels and induction of UDP-GT by TCDD, 3,3',4,4',5-pentachlorobiphenyl (PCB 126) or 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) in a 13 week feeding study in female rats (Van Birgelen *et al.*, 1995).

Nonplanar PCBs and especially OH-PCBs, which do not bind to the AhR and are not able to induce UDP-GT, have their thyroid effects mediated through several other pathways. The most pronounced effects of OH-PCBs on the thyroid hormone system are the binding to transthyretin (TTR), a thyroid hormone transport protein which forms a complex with retinol binding protein (RBP). Exposure of rats to 3,3',4,4'-tetrachlorobiphenyl (PCB 77) resulted in marked reductions of plasma thyroxine levels and retinol (Brouwer and Van den Berg, 1986). Further investigation showed a selective retention of a hydroxylated metabolite (4-OH-3,3',4',5-tetrachlorobiphenyl) in plasma of exposed rats which was able to bind to TTR (Brouwer *et al.*, 1990). The competitive binding of this metabolite to TTR resulted in thyroxine displacement and disturbance of the TTR-RBP complex leading to increased glomerular filtration of RBP (Brouwer and Van den Berg, 1986). Exposure of pregnant mice to the same parent compound resulted in a high and selective accumulation of 4-OH-3,3',4',5-tetrachlorobiphenyl in fetal mouse plasma and reductions in thyroid hormone levels (Darnerud *et al.*, 1996). The binding of the metabolite to TTR was confirmed in both fetal and maternal plasma, and it was suggested that binding of OH-PCBs and also other phenolic organohalogen compounds to TTR *in vivo* may result in facilitated transport of OH-PCBs over the placenta to the fetal compartment, leading to decreased thyroid hormone levels in the maternal but especially the fetal compartment with possible consequences on fetal brain development. Administration of the commercial PCB mixture Aroclor 1254 to pregnant rats from gestation days 10

to 16 resulted in a selective accumulation of another PCB-metabolite (4-OH-2,3,3',4',5-pentaCB, 4-OH-CB107) in fetal plasma and brain, accompanied by low concentrations of T_4 in dams and fetuses at GD20 and in neonates at PND4 and PND21 (Morse *et al.*, 1996a), and long term effects on several neurochemical markers (Morse *et al.*, 1996b) and brain serotonin metabolism in the exposed offspring (Morse *et al.*, 1996c). This PCB-metabolite is one of the major metabolites detected in human blood (Bergman *et al.*, 1994; Letcher *et al.*, 2000; Sandau *et al.*, 2000).

The binding of OH-PCBs to TTR was further elucidated in *in vitro* competition binding studies using purified human TTR (Lans *et al.*, 1993, 1994; McKinney *et al.*, 1985; Rickenbacher *et al.*, 1986). The affinities of OH-PCB congeners for TTR *in vitro* are up to 10 times higher compared to thyroxine itself (Lans *et al.*, 1993). Also other organohalogen substances, such as halogenated phenols, can bind to TTR (Van den Berg, 1990). The presence of a hydroxy-group at the *para* or *meta* positions of the phenyl ring of PCBs, with at least one chlorine substitution at an adjacent position was thought to be an essential prerequisite for TTR binding (Lans *et al.*, 1993). This was confirmed by X-ray crystallography studies with 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (Lans, 1995). However, Chauhan *et al.* (1998) showed that also parent compounds were capable of binding to TTR, although with weak affinity. All OH-PCBs determined in human blood were shown to have high affinities for TTR *in vitro* (Lans *et al.*, 1993).

Another mechanism of action of OH-PCBs on the thyroid hormone system is their effects on metabolism. OH-PCBs can influence thyroxine metabolism by a strong inhibition of thyroxine sulfation, which has been shown *in vitro* (Schuur *et al.*, 1998, 1999). Since sulfation is a major regulation pathway of T_4 in the fetus, the interference of OH-PCBs with thyroid hormone sulfation may have consequences for the development of the fetus, and in particular for fetal brain development (Brouwer *et al.*, 1998). Another effect of OH-PCBs on thyroxine metabolism is mediated via the inhibition of deiodinase activity, thereby preventing the formation of the active thyroid hormone 3,3',5-triiodothyronine (T_3; Adams *et al.*, 1990; Lans, 1995; Rickenbacher *et al.*, 1989).

The thyrotoxic effects of PCBs have been suggested to form the basis for the developmental neurotoxicity observed following prenatal PCB exposure (Porterfield and Hendrich, 1993). Prenatal exposure to PCBs is known to result in numerous behavioural alterations including impaired learning and memory, altered activity levels, delayed development of reflexes, impaired acquisition of active avoidance tasks, and delayed development of auditory startle and air righting reflex (reviewed in Juarez de Ku and Meserve, 1994). For example, mice exposed *in utero* to the dioxin-like PCB77 have demonstrated spinning behaviour, hyperactivity and impaired acquisition of avoidance response (Tilson *et al.*, 1979), and mice exposed during the early neonatal period exhibited depression of spontaneous motor behaviour (Eriksson *et al.*, 1991). Effects of prenatal exposure to PCBs on hearing deficits were also reported (Goldey *et al.*, 1995a). These hearing deficits could be partially prevented by T_4 replacement (Goldey and Crofton, 1998).

Interactions of PCBs and OH-PCBs with the estrogen system

The estrogenic activities of commercial PCB mixtures administered to rats was already determined in the 1970s (Bitman and Cecil, 1970). 4-OH-2',4',6'-trichlorobiphenyl and 4-OH-2',3',4',5'-tetrachlorobiphenyl were estrogenic in a rodent uterotrophic assay and were able to bind competitively to the estrogen receptor (Korach *et al.*, 1988). However, these OH-PCBs were not environmentally relevant, since they have not been identified as persistent, retained metabolic products in human or wildlife blood samples. The OH-PCBs identified in human serum were mostly weakly anti-estrogenic (Moore *et al.*, 1997). Recently, Kester *et al.* (2000) showed that several OH-PCBs are extremely potent inhibitors of the human estrogen sulfotransferase (hEST) enzyme *in vitro*. The OH-PCB with the highest inhibition potency was 4-OH-CB107. Estrogen sulfation is a normal route of reversible inactivation of estradiol. As a result of the inhibition of estrogen sulfation, OH-PCBs may increase the bioavailability of E_2 in target tissues, thereby exerting an indirect estrogenic effect.

PBDEs and OH-PBDEs

At the beginning of the research described in this thesis, very little was known about the toxicity of PBDEs. Most of the studies with PBDEs were carried out using technical mixtures of which the purity was known in several cases, but the isomer composition was unknown. The available data showed no severe signs of toxicity in subacute and subchronic toxicity studies with deca-, octa- and pentaBDE preparations (IPCS, 1994; Norris *et al.*, 1975a, 1975b). The target organs for toxicity of PBDEs were determined to be the liver, kidney and thyroid gland, which were enlarged and/or showed minor histopathological changes (IPCS, 1994). The most sensitive end point of PBDE toxicity *in vivo* appears to be effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production (i.e. lowering of free and total T_4 concentrations) in rats and mice (Darnerud and Sinjari, 1996, Fowles *et al.*, 1994). PBDEs were also shown to induce cytochrome P450 1A1 and 1A2 *in vitro* (Hanberg *et al.*, 1991) and *in vivo* (Von Meyerinck *et al.*, 1990), thus suggesting that several PBDEs are able to act via the AhR mediated signal transduction pathway. The level of CYP1A1/1A2 induction in rats of the commercial pentaBDE mixture Bromkal 70 was the same as observed after Aroclor 1254 treatment (both given as a single dose of 300 mg/kg body weight). However, it is suggested that this dioxin-like activity may be partly attributed to possible impurities of brominated dioxin or furan compounds present in these mixtures (Darnerud *et al.*, 2001).

Possible adverse effects of endocrine active organohalogen compounds on human health

The presence of organohalogen compounds in the environment with potential endocrine activity has lead to the hypothesis that exposure to these chemicals might alter the endocrine system, causing adverse developmental and reproductive effects in both animals and humans. However, human information on developmental reproduction and sexual behavioural effects of organohalogen compounds is scarce. The most well documented effects of organohalogen compounds on humans are neurodevelopmental effects. Clear evidence of developmental toxicity of dioxin-like compounds was observed in two cohorts (the Yusho and Yu-Cheng cohort) that were inadvertently exposed to

complex mixtures of PCBs and PCDFs (Kuratsune, 1989; Rogan *et al.*, 1988). High exposed infants showed delays in attaining developmental milestones, and at the age of 8 to 13 years behavioural problems, intellectual deficits and growth retardation were evident (Chen *et al.*, 1992; Peterson *et al.*, 1993). There were also sex-related effects, e.g. prenatally exposed boys had unusually high serum levels of estrogen, and had penises that are significantly smaller at puberty than those of age-matched controls (Guo *et al.*, 1993).

Exposure of human infants to background environmental levels of PCBs can also lead to subtle changes in development. Poorer psychomotor performance, poorer visual recognition memory and poorer performances in memory scales test have been reported in infants exposed to relatively high levels of PCBs (Gladen *et al.*, 1988; Sauer *et al.*, 1994; Jacobson *et al.* 1985, 1990; reviewed by Brouwer *et al.*, 1995). There were also indications that increasing levels of PCBs in mother's milk correlated with subtle decreases in thyroid hormone levels in human infants (Koopman-Esseboom *et al.*, 1994; Nagayama *et al.*, 1996). It may thus be possible that background exposure to endocrine active (especially thyroidogenic) organohalogen compounds in humans can lead to subtle changes in the human endocrine system, thereby altering neurodevelopment in human infants. There is a good resemblance between neurodevelopmental changes observed following background exposure to e.g. PCBs and changes caused by a prenatal or early postnatal hypothyroid status (reviewed by Brouwer *et al.*, 1998).

Outline of the thesis

The aim of the research described in this thesis was to investigate the possible endocrine activity of newly identified organohalogen substances in human blood (the polybrominated diphenyl ethers, PBDEs), and to predict the consequences of human exposure to endocrine active, especially phenolic organohalogens (e.g. hydroxylated metabolites of PCBs or PBDEs) on fetal and neonatal development. It was hypothesised that the toxicity profile of PBDEs would resemble the profile of PCBs. In addition, it was also hypothesised that the presence of several hydroxylated PCBs and PBDEs in human blood could be explained by their selective retention in the blood due to binding to human transthyretin, and that *in vivo* exposure of pregnant females to these phenolic organohalogens would lead to the transport of the compounds over the placenta and exposure of the unborn. This may result into long term adverse effects on the offspring.

The possible interference of PBDEs and related brominated flame retardants with the endocrine system was investigated *in vitro* (described in part I). The ability of these compounds to bind to human TTR was determined using an *in vitro* T_4-TTR competition binding assay (*Chapter 2*). Structural requirements elucidated from these studies were compared with the known structure activity relationships of related compounds. The possible estrogenic or anti-estrogenic activities of PBDEs and OH-PBDEs are presented in *Chapter 3*. Differently brominated bisphenol A compounds were included in these studies to investigate the structure activity relationship for thyroidogenicity (*Chapter 2*) and estrogenicity (*Chapter 3*) in more detail. In *Chapter 4*, the toxicity of PBDEs is summarized and compared with the mechanism of action of PCBs. The molecular interaction of two brominated phenols with transthyretin, determined in a collaborative study (Ghosh *et al.*, 2000) using

X-ray crystallography, is also discussed. In addition, the role of metabolites is discussed, and the choice for the model compound for *in vivo* studies is clarified.

To be able to predict the possible adverse effects of human exposure to hydroxylated PCB or PBDE compounds, *in vivo* toxicity studies were performed. In part II *in vivo* studies are described that were performed with a model compound, the PCB metabolite 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107), which was already detected in relatively high quantities in human blood (described in part II). The uptake and distribution of 4-OH-CB107 was investigated in pregnant female rats and their fetuses (*Chapter 5*). Finally, the long-term effects of *in utero* exposure to 4-OH-CB107 on neonatal development, reproductive function, steroid hormone levels, behaviour and hearing in rat offspring were compared with effects induced by the parent compound (*Chapters 6* and *7*). In *Chapter 8* the *in vivo* effects are summarized. The overall impact and conclusions of the results described in this thesis are presented in *Chapter 9*.

PART I

In vitro studies

CHAPTER 2

Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro

Abstract

Brominated flame retardants such as polybrominated diphenyl ethers (PBDEs), pentabromophenol (PBP), and tetrabromobisphenol A (TBBPA) are produced in large quantities for use in electronic equipment, plastics, and building materials. Because these compounds have some structural resemblance with the thyroid hormone thyroxine (T_4), it was suggested that they may interfere with thyroid hormone metabolism and transport, e.g., by competition with T_4 on transthyretin (TTR). In the present study, we investigated the possible interaction of several brominated flame retardants with T_4 binding to TTR in an *in vitro* competitive binding assay, using human TTR and ^{125}I-T_4 as the displaceable radioligand. Compounds were tested in at least eight different concentrations ranging from 1.95 to 500 nM. In addition, we investigated the structural requirements of these and related ligands for competitive binding to TTR. We were able to show very potent competition binding for TBBPA and PBP (10.6- and 7.1-fold stronger than the natural ligand T_4, respectively). PBDEs were able to compete with T_4-TTR binding only after metabolic conversion by induced rat liver microsomes, suggesting an important role for hydroxylation. Brominated bisphenols with a high degree of bromination appeared to be more efficient competitors, whereas chlorinated bisphenols were less potent compared to their brominated analogues. These results indicate that brominated flame retardants, especially the brominated phenols and tetrabromobisphenol A, are very potent competitors for T_4 binding to human transthyretin *in vitro* and may have effects on thyroid hormone homeostasis *in vivo* comparable to the thyroid-disrupting effects of PCBs.

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Introduction

It is well established that several classes of environmental contaminants can affect thyroid gland morphology and hormonal status (for reviews see Brouwer *et al.*, 1998; Brucker-Davis, 1998), but the exact mechanisms of interference are not fully understood. There are at least three different levels at which organohalogen contaminants are known to interact with the thyroid hormone system: at the thyroid gland, in thyroid hormone metabolism, and with thyroid hormone transport proteins. A number of chemicals have been reported to bind to transthyretin (TTR), one of the thyroid hormone-binding transport proteins in plasma of vertebrate species. In particular, metabolites of the polyhalogenated aromatic hydrocarbons (PHAHs) such as hydroxylated polychlorinated biphenyls (OH-PCBs), hydroxylated polychlorinated dibenzo-*p*-dioxins (OH-PCDDs), and pentachlorophenol (PCP) have been shown to bind to TTR in *in vitro* and/or *in vivo* studies (Brouwer *et al.*, 1988; Lans *et al.*, 1993; McKinney and Waller, 1994; van den Berg *et al.*, 1991; van Raaij *et al.*, 1991). It is hypothesized that the binding of chemicals to TTR, thereby displacing the natural ligand 3,3',5,5'-tetraiodothyronine (thyroxine, T_4), leads to an increase in the clearance of T_4 and a decrease in serum T_4 concentrations (Darnerud *et al.*, 1996), a common feature in animals that have been exposed to PHAHs (Brouwer *et al.*, 1998; Brucker-Davis, 1998).

The research on chemicals binding to transthyretin has been focused mainly on the polychlorinated dibenzo-*p*-dioxins (PCDDs) and biphenyls (PCBs), i.e., compounds that have been banned or are under control measures for further environmental reduction. Hydroxylated PCBs, especially those with a hydroxy group on *meta* or *para* positions with one or more adjacent halogen substituents, have been shown to be potent ligands for TTR (Lans *et al.*, 1993; Rickenbacher *et al.*, 1986) because of their structural resemblance with thyroxine. Other organohalogen compounds that are extensively used at the moment, particularly the brominated flame retardants tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDEs) (TemaNord, 1998), show an even closer structural relationship to thyroxine than the PCBs. Therefore, the possibility exists that these brominated flame retardants interact with TTR and other aspects of thyroid hormone metabolism. Of the brominated flame retardants in use today, about one-third are polybrominated diphenyl ethers (PBDEs), another one-third are tetrabromobisphenol A and derivatives, and the last third is composed of a variety of bromine-containing products, including polybrominated biphenyls (PBBs) (OECD, 1994). The production volume of TBBPA in 1995 was approximately 60,000 tonnes per year (IPCS, 1995). Its primary use is as a reactive intermediate in the production of flame-retarded epoxy resins used in printed circuit boards (IPCS, 1995). PBDEs are extensively used as flame retardants in plastics, paints, electrical components, and synthetic textiles (IPCS, 1994). They have been produced in large quantities since the 1980s, mostly as commercial mixtures such as Bromkal 70-5DE. TBBPA has been detected in sediment samples in Japan in concentrations of 0.5-140 µg/kg dry weight (Watanabe *et al.*, 1983), but it is not normally detected in environmental biologic samples (IPCS, 1995).

PBDEs have been found in various biotic samples, such as fish-eating birds and marine mammals (Jansson *et al.*, 1987), shellfish and sediment (Haglund *et al.*, 1997), and even in human blood (Sjödin *et al.*, 1999) and breast milk (Meironyté *et al.*, 1999). So far, the PBDE concentrations detected in wildlife and humans are lower than the concentrations of PCBs. However, Meironyté *et al.* (1999) showed that PBDE concentrations in human milk sampled in Sweden from 1972 to 1998 increased from 0.07 to 4.02 ng/g lipid weight. Hydroxylated and methoxylated PBDEs (OH-PBDEs and MeO-PBDEs) have also been detected in various biotic samples from the Baltic Sea (Asplund *et al.*, 1999; Haglund *et al.*, 1997). Concentrations of the OH-PBDEs in blood plasma from Baltic salmons were estimated to be about 30-50 ng/g lipid weight, similar to concentrations of the major PBDEs in these samples (Asplund *et al.*, 1999).

Because of the high production volume of brominated flame retardants, the presence of these compounds in biotic samples, and their close structural resemblance with thyroxine, we examined the ability of several of these compounds and their metabolites (in the case of PBDEs) to bind to human TTR by an *in vitro* T_4-TTR competition binding assay (Lans *et al.*, 1993). Because no hydroxylated PBDEs were available at the time for this study, a method was designed to include microsomal activation in the T_4-TTR competition binding assay. For comparison, three synthesized hydroxylated PBDEs with structural resemblance to the thyroid hormones 3,5-diiodothyronine (T_2), 3,3',5-triiodothyronine (T_3) and 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) were also tested for their potency to compete with T_4-TTR binding. The resulting structure-activity relationships were compared with the known structure-activity relationships of related (especially chlorinated) compounds.

Materials and methods

Chemicals

All chemicals were of > 98% purity unless otherwise stated. 2,4,6-Tribromoaniline, 2,4-dibromophenol (2,4-DBP; 95%), 2,3,5,6-tetrabromo-*p*-xylene, 2,3,4,5,6-pentabromotoluene, brominated bisphenol A diglycidyl ether, tetrabromobisphenol A (TBBPA, 97%), tetrachlorobisphenol A (TCBPA), pentabromophenol (PBP, 96%), bisphenol A (97%), 4-phenoxyphenol, and hexabromobenzene were obtained from Aldrich Chemical Company (Bornem, Belgium). Bisphenol A diglycidylether, bisphenol A bis(2,3-dihydroxypropyl)ether, bisphenol A bis(3-chloro-2-hydroxypropyl)ether, 2,4,6-tribromophenol (TBP), and phenobarbital (PB) were purchased from Fluka Chemie (Buchs, Switzerland). 2-Hydroxy-2',4,4'-trichlorodiphenyl ether was from Ultra Science (N. Kingstown, RI).

Pure PBDE congeners (> 98% pure) were synthesized as described elsewhere (Marsh *et al.*, 1999; Örn *et al.*, 1996). Three hydroxylated brominated diphenyl ethers, 4-(2,4,6-tribromophenoxy)phenol, 2-bromo-4-(2,4,6-tribromophenoxy)phenol and 2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol, were synthesized as described by Marsh *et al.* (1998) and were at least 99% pure. Monobromobisphenol A (MBBPA, containing 96.5% monobromobisphenol A and 3.5% dibromobisphenol A), dibromobisphenol A (diBBPA, containing 99.4% dibromo- and 0.6% tribromobisphenol A), and tribromobisphenol A (triBBPA, 100% pure) were synthesized by

bromination of bisphenol A using bromine in acetic acid at room temperature (Sara Rahm, unpublished).

^{125}I-L-3',5'-Thyroxine (spec. act. 46 μCi/μg) was purchased from Orange Medical (Tilburg, The Netherlands). Human prealbumin (transthyretin, TTR, 98% pure), clofibrate (CLOF), and 3,3',5,5'-L-thyroxine (T_4) were obtained from Sigma Chemical Company, St. Louis, MO. Tris, saccharose, methanol, ethanol, dichloromethane and diisopropyl ether (all analytical grade) were from Merck Chemical Company (Darmstadt, Germany). Biogel P-6DG desalting gel was obtained from Bio-Rad Laboratories (Richmond, CA). β-Naphthoflavone (β-NF), EDTA, and dimethylsulfoxide (99.9% pure) were obtained from Janssen Chimica (Geel, Belgium). NADPH was obtained from Boehringer (Mannheim, Germany).

Bisphenol A and mono- and dibromobisphenol A were dissolved in ethanol and stored at –20°C. Only 2,3,5,6-tetrabromo-*p*-xylene and 2,3,4,5,6-pentabromotoluene were dissolved in dichloromethane because they were not soluble in ethanol or dimethylsulfoxide (DMSO). All other compounds were dissolved in DMSO.

Preparation of microsomes

Nine male Wistar WU rats (14 weeks of age) were purchased from Charles River (Sulzfeld, Germany) and allowed to acclimatize for 2 weeks. They were kept in an artificial light-dark cycle (06:00 lights on, 18:00 h lights off), with room temperature at 21 ± 1°C and humidity at 50 ± 10%. Animals were provided rat chow (Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. To induce microsomes, three rats per group that were naïve to chemical treatment were pretreated with β-naphthoflavone (β-NF, three daily ip injections of 30 mg/kg body weight dissolved in corn oil), phenobarbital (PB, 0.1% w/v in the drinking water for 7 days), or clofibrate (CLOF, four daily oral administrations of 200 mg/kg bw). One day after the last treatment, the rats were sacrificed under ether anaesthesia and the livers were removed. All procedures were approved by the Animal Welfare Committee of Wageningen University. Livers of rats from each treatment group were pooled and homogenized in ice-cold 0.1 M Tris-HCl buffer, pH 7.5 (3 ml/g liver), containing 0.25 M sucrose, using a Potter-Elvehjem tube and Teflon pestle. The homogenate was centrifuged for 30 minutes at 9,000 × *g* (4°C). The resulting supernatant was centrifuged at 105,000 × *g* and 4°C for 90 min. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.5). Microsomes were stored in aliquots of 1 ml at –80°C until use. Protein concentrations were determined using the Bio-Rad Coomassie blue assay (Bio-Rad, Richmond, CA), using BSA as a standard (Bradford, 1976).

Metabolism of PBDEs in vitro

As almost no hydroxylated PBDEs have been synthesized so far, 17 PBDE congeners were metabolized by incubation with induced hepatic microsomes, as described for PCBs (Morse *et al.*, 1995) with slight modifications. Briefly, 10 μM of each PBDE congener was incubated with 1 mg/ml hepatic microsomes in a 0.1 M Tris-HCl buffer (pH 7.5) in a total volume of 2 ml. After preincubation for 2 min in a shaking water bath at 37°C, the reaction was initiated with NADPH (1 mM). Metabolism was stopped after 30 min by the addition of 2 ml ice-cold methanol. After centrifugation, the supernatants were extracted twice with 2 ml diisopropyl ether by vortexing for 30s,

centrifugation at 1000 × *g* for 5 min, and then removal of the diisopropyl ether phase. The ether extracts were pooled, dried under nitrogen, and stored at 4°C until further analysis (but not longer than 1 week). Control incubations were carried out by performing identical incubations with the PBDE without the addition of NADPH. For determining the possible background of T_4-TTR competition by microsomal extracts, incubations were also carried out without the addition of a PBDE to the microsomes. The extracts were dried by evaporation, and residues were dissolved in 50 µl methanol prior to the T_4-TTR competition binding studies.

In vitro T_4-TTR competition binding studies

The analysis of the capacity of various compounds to compete with T_4 binding to TTR was performed as described previously (Lans *et al.*, 1993), with modifications. The assay mixture was a 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1 mM EDTA, 30 nM human TTR, a mixture of ^{125}I-labeled and unlabeled T_4 (70,000 cpm, 55 nM), and competitors (cold T_4, pure compounds or extracts) with increasing concentrations (at least eight different concentrations), in a total volume of 200 µl. Control incubations contained 5 µl ethanol, methanol, DMSO, or dichloromethane (depending on the solvent used) instead of competitor. Total ^{125}I-radioactivity added to each of the incubation mixtures was checked by gamma counting (Multi Prias, Packard Instrument Co., Meriden, CT). The incubation mixtures were allowed to reach binding equilibrium overnight at 4°C. After incubation, protein-bound and free ^{125}I-T_4 were separated on 1 ml Biogel P-6DG columns and spin-force eluted with 200 µl Tris-HCl buffer (1 min at 100 × *g* in a precooled centrifuge, Difuge, Hereaus) to reduce transit time on the column (about 30 s) in order to minimize possible dissociation of the complex (Somack *et al.*, 1982). Radioactivity was determined in the eluate containing the protein-bound ^{125}I-T_4-fraction and compared to control incubations. Nonspecific binding was also determined in each series of experiments by addition of 10 µM cold T_4 and was less than 10%.

In the case of pure compounds, the competitors were first diluted in 0.1 M Tris-HCl buffer (pH 8.0, containing 0.1 M NaCl, 1 mM EDTA) and added to the assay mixture in a concentration series from 10^{-9} to 10^{-4} M. To study possible competition binding of PBDE metabolites, extracts of microsomal incubations were diluted 0, 3, or 9 times in methanol and 5 µl was added to the incubation mixture as competitor. The maximum concentration of metabolites formed could thus be no more than 250 nM with 100% conversion. The maximum percentage of solvent in the assay mixture did not exceed 0.5%. Control incubations with microsomal extracts without PBDEs were used to determine possible background competition, whereas microsomal extracts from incubations with PBDEs but without NADPH were used to determine possible competition by the parent compound itself.

Competition binding curves for pure compounds were made by plotting relative ^{125}I-T_4-protein binding (% of control) against concentration competitor. For microsomal extracts, competition binding curves were made by plotting relative ^{125}I-T_4-protein binding (% of control, with control incubations of microsomes set to 100%) against the dilution factor, as no reference PBDE-metabolites are presently available.

Table 2.2 *In vitro* competition of ^{125}I-T_4-TTR binding by several brominated flame retardants and related compounds.

Compound	IC_{50} (nM)[a]	Relative Potency[b]	K_a ($*10^7$ M^{-1})[c]	Maximum competition (%)[d]	Highest tested concentration (nM)
Thyroxine (T_4)	80.7	1	3.50 ± 3.0	75 ± 3.7	500
Hexabromobenzene	n.d.	n.d.	n.d.	9.1 ± 2.1	5000
2,4,6-tribromoaniline	n.d.	n.d.	n.d.	6.9 ± 3.3	30000
2,4-Dibromophenol (2,4-DBP)	1.4 (± 0.2) x 10^3	0.06 (± 1) x 10^{-3}	0.15 ± 0.06	50 ± 1.8	25000
2,4,6-Tribromophenol (2,4,6-TBP)	67.2 ± 2.7	1.20 ± 0.05	4.30 ± 0.2	87.4 + 8.6	500
Pentabromophenol (PBP)	11.5 ± 1.8	7.14 ± 1.11	25.6 ± 4.0	96.2 ± 4.3	500
2,3,5,6-Tetrabromo-p-xylene	n.d.	n.d.	n.d.	1.9 ± 0.3	5000
2,3,4,5,6-Pentabromo-toluene	n.d.	n.d.	n.d.	2.1 ± 0.1	5000
Bisphenol A	n.d.	n.d.	n.d.	7.3 ± 1.9	500
Monobromobisphenol A (MBBPA)	n.d.	n.d.	n.d.	11.4 ± 2.5	500
Dibromobisphenol A (DiBBPA)	n.d.	n.d.	n.d.	18.6 ± 4.6	500
Tribromobisphenol A (triBBPA)	140.0 ± 17.5	0.58 ± 0.07	2.08 ± 0.3	53.5 ± 1.8	500
Tetrabromobisphhenol A (TBBPA)	7.7 ± 0.9	10.6 ± 1.29	37.9 ± 4.7	96.5 ± 0.1	500
Tetrachlorobisphenol A (TCBPA)	106.8 ± 10.3	0.76 ± 0.07	0.19 ± 0.1	62.3 ± 0.7	500
Bisphenol A diglycidyl ether	n.d.	n.d.	n.d.	1.1 ± 1.0	5000
Bisphenol A diglycidyl ether, brominated	n.d.	n.d.	n.d.	1.2 ± 1.0	5 mg[e]
Bisphenol A bis(2,3-dihydroxy-propyl)ether	n.d.	n.d.	n.d.	0.5 ± 0.2	5000
Bisphenol A bis(3-chloro-2-hydroxy-propyl)ether	n.d.	n.d.	n.d.	1.0 ± 0.5	5000

Results are presented as means ± SD of (at least) triplicate measurements
[a] concentration of competitor at 50% competition
[b] calculated as ratio of IC_{50} (T_4)/ IC_{50} (competitor)
[c] Binding affinity constants as determined by the Ligand program
[d] Maximum percentage of competition reached at highest tested concentration
[e] Molecular weight is unknown because of variable bromination.
n.d. = not determined, no displacement observed within the tested range.

In addition, for the bisphenol A analogues, the TTR binding potency increased with a higher level of bromination. The potency of triBBPA was 18 times less compared to TBBPA, whereas no or only slight competition was observed with di-, mono- and nonbrominated bisphenol A (approximately 19, 11 and 7% competition reached at 500 nM, respectively, Table 2.2). Interestingly, replacing the bromine atoms by chlorine atoms in the bisphenol A core structure (e.g., tetrabromobisphenol A versus tetrachlorobisphenol A [TCBPA]) resulted in an almost 14 times lower TTR-binding competition potency (IC_{50} values of TCBPA and TBBPA were 106.8 ± 10.3 and 7.7 ± 0.9, respectively). The competition binding curves of diBBPA, triBBPA, TBBPA and TCBPA are given in Figure 2.1.

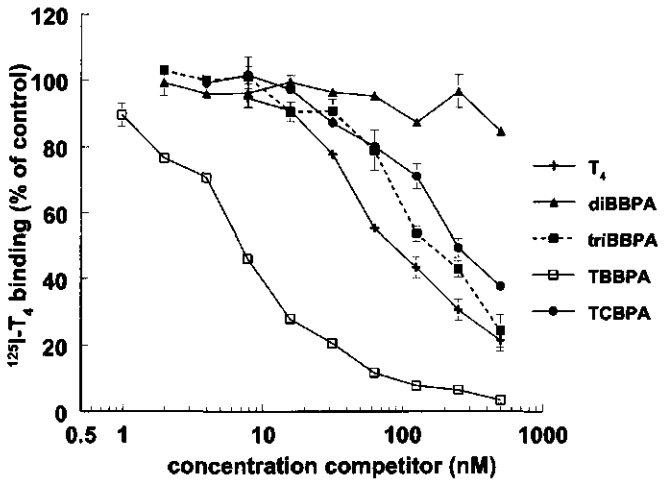

Figure 2.1. Displacement of T_4 from TTR by halogenated bisphenol A congeners. Data points are mean values ± SD of one representative measurement in duplicate. If no error bar is visible, it is smaller than the marker. Relative ^{125}I-T_4-TTR binding is presented as percentage of control value. Abbreviations used: diBBPA, dibromobisphenol A; triBBPA, tribromobisphenol A; TBBPA, tetrabromobisphenol A; TCBPA, tetrachlorobisphenol A.

T_4-TTR binding competition with microsomal extracts of PBDEs

In total, 17 different PBDE congeners (see Figure 2.2 for their structure) were tested before and after incubation with differently induced hepatic microsomes for T_4-TTR competition binding potency by their possible metabolites formed. Because no reference PBDE metabolites were available at the time of this study, the competition potency of microsomal extracts could be investigated only by dilution technique. In Figure 2.3, a representative example of the T_4-TTR competition binding by microsomal extracts is given. No competition of T_4-TTR was observed with control microsomal incubations without NADPH (Fig. 2.3 [A and B], triangles) or without PBDE (Fig. 2.3 [A and B], circles), indicating that microsomes did not cause background competition and parent PBDEs were not able to bind to TTR.

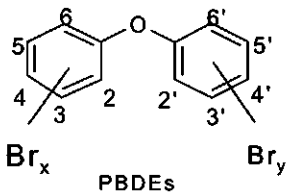

Figure 2.2. Core structure of polybrominated diphenyl ethers (PBDEs).

Table 2.3. In vitro T_4-TTR competition binding of extracts from polybrominated diphenyl ethers after incubation with liver microsomes enriched with CYP1A, CYP2B or CYP4A3.

PBDE	bromine substitution	phenobarbital microsomes (CYP2B)	∃-naphthoflavone microsomes (CYP1A)	Clofibrate microsomes (CYP4A3)
15	4,4'	++	++	–
28	2,4,4'	++	+	+
30	2,4,6	++	++	++
32	2,4',6	–	+	+
47	2,2',4,4'	++	–	–
51	2,2',4,6'	++	–	+
71	2,3',4',6	+	+	+
75	2,4,4',6	++	+	+
77	3,3',4,4'	++	+	+
85	2,2',3,4,4'	+	–	–
99	2,2',4,4',5	+	–	–
100	2,2',4,4',6	++	–	–
119	2,3',4,4',6	++	–	–
138	2,2',3,4,4',5'	–	–	–
153	2,2',4,4',5,5'	–	–	–
166	2,3,4,4',5,6	+	–	–
190	2,3,3',4,4',5,6	–	–	–

Results are given as qualitative data: – = no competition with T_4-binding, + = 20-60% competition, ++ = more than 60% competition with T_4 in comparison with control incubations (without NADPH or without compound).

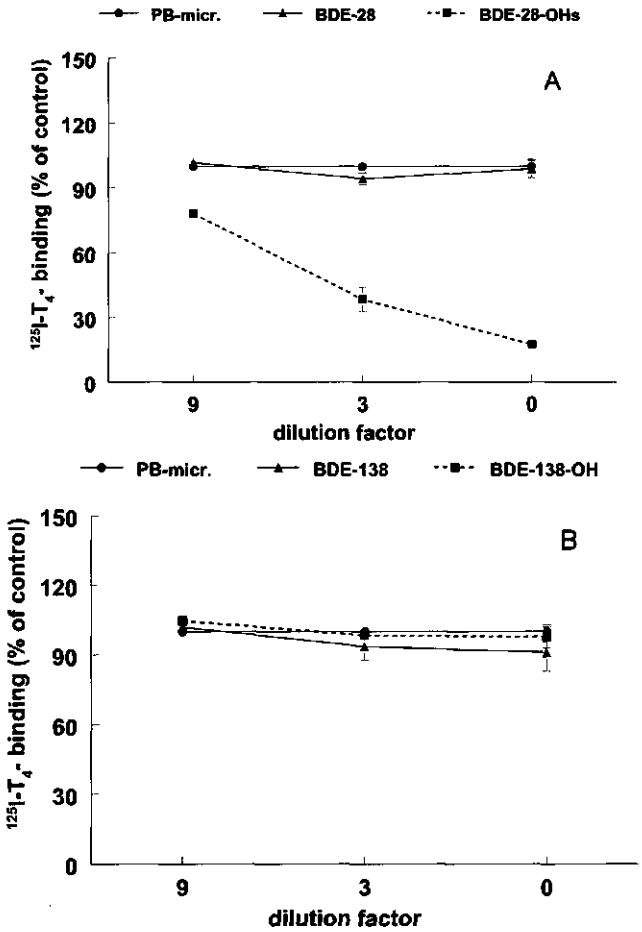

Figure 2.3. T_4-TTR competition binding of 2,4,4'-triBDE (BDE-28) (A) and 2,2',3,4,4',5'-hexaBDE (BDE-138) (B) prior to microsomal transformation with Phenobarbital (PB)-induced rat microsomes (triangles) and of PBDE-metabolites (squares) after microsomal transformation with Phenobarbital (PB)-induced rat microsomes. Data present mean ± SD. PB-micr., Phenobarbital-induced microsomes (circles).

Incubation of PBDEs with PB microsomes (mostly P450 2B enriched) in the presence of NADPH resulted in the formation of metabolites that were able to compete with T_4 binding to TTR, with the exception of incubation extracts from 2,4',6-triBDE (BDE 32); 2,2',3,4,4',5'-hexaBDE (BDE 138, Fig. 2.3B); 2,2',4,4',5,5'-hexaBDE (BDE 153); and 2,3,3',4,4',5,6-heptaBDE (BDE 190) (Table 2.3). P450 1A, P450 2B, and P450 4A3 enriched microsomes all catalyzed the formation of TTR-binding metabolites from 2,4,6-triBDE (BDE 30); 2,3',4',6-tetraBDE (BDE 71); 2,4,4',6-tetraBDE (BDE 75); and 3,3',4,4'-tetraBDE (BDE 77).

Hydroxylated PBDEs (OH-PBDEs)

Three pure hydroxylated PBDEs (OH-PBDEs), synthesized for their structural resemblance with the thyroid hormones 3,5-diiodothyronine (3,5-T_2), 3,3',5-triiodothyronine (T_3), and 3,3',5,5'-tetraiodothyronine (T_4), were tested in the T_4-TTR competition binding assay (Table 2.4, Figure 2.4). The relative potencies showed that the T_4-like (2,6-dibromo-4-[2,4,6-tribromophenoxy]phenol) and T_3-like (2-bromo-4-[2,4,6-tribromophenoxy]phenol) OH-PBDEs were 1.42- and 1.22-fold more potent, respectively, than T_4, and the percentage competition at 500 nM exceeded that of the natural ligand (Table 2.4). 4-Phenoxyphenol and 2-hydroxy-2',4,4'-trichlorodiphenyl ether showed no interaction with human TTR.

Table 2.4. In vitro T_4-TTR competition binding by synthetic polybrominated diphenyl ether metabolites.

Compound	IC_{50} (nM) [a]	Relative Potency [b]	K_a ($* 10^7 M^{-1}$) [c]	Max. competition (%) [d]	Highest tested conc. (nM)
Thyroxine (T_4)	80.7	1	3.50 ± 0.3	75 ± 3.7	500
4-Phenoxyphenol	n.d.[d]	n.d.	n.d.	10.3 ± 0.2	5000
T_2-OH-BDE	199.2 ± 12.3	0.41± 0.02	1.28 ± 0.33	38.2 ± 4.3	500
T_3-OH-BDE	66.0 ± 0.98	1.22 ± 0.02	4.38 ± 0.6	86.1 ± 5.0	500
T_4-OH-BDE	57.0 ± 2.3	1.42 ± 0.06	5.09 ± 0.20	91.8 ± 4.0	500
2-OH-2',4,4'-trichlorodiphenyl ether	n.d.	n.d.	n.d.	2.1 ± 0.3	500

Note. Results are presented as means ± SD of triplicate measurements.

[a] concentration of competitor at 50% competition

[b] calculated as ratio of IC_{50} (T_4)/ IC_{50} (competitor)

[c] Binding affinity constants as determined by the Ligand program

[d] Maximum percentage of competition reached at highest tested concentration

n.d. = not determined, no displacement observed within the tested concentrations

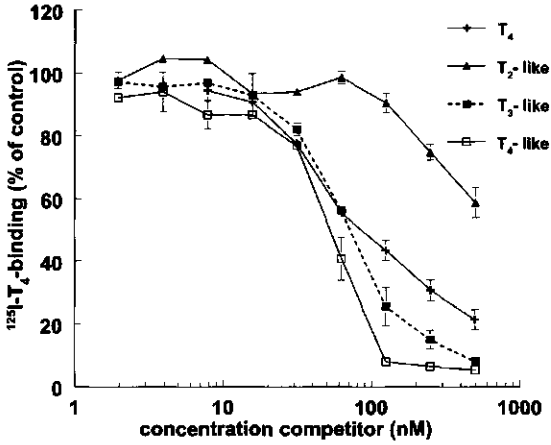

Figure 2.4. Displacement of T_4 from TTR by hydroxylated polybrominated diphenyl ethers resembling thyroid hormones. Data points are mean values ± SD of one representative measurement in duplicate. If no error bar is visible, it is smaller than the marker. Relative ^{125}I-T_4-TTR binding is presented as percentage of control value. Abbreviations: T_2-like = 4-(2,4,6-tribromophenoxy)-phenol; T_3-like = 2-bromo-4-(2,4,6-tri-bromophenoxy)phenol; T_4-like = 2,6-di-bromo-4-(2,4,6-tribromo-phenoxy)phenol.

Discussion

The results presented in this study clearly demonstrate for the first time that hydroxylated brominated flame retardants of several different classes are able to bind to human transthyretin *in vitro*, some with extremely high potency, e.g., TBBPA and PBP. This is an important finding, as brominated flame retardants are used extensively at present for a large variety of applications and can be detected in wildlife and humans (Bergman *et al.*, 1999a; Meironyté *et al.*, 1999; IPCS, 1997). The results of this paper thus indicate the possible capability of a large group of particularly brominated industrial chemicals to interfere with and potentially disrupt thyroid hormone transport and metabolism.

The structure-affinity data of brominated (bis)phenols that can be deduced from this study are in good agreement with previous studies on several industrial chemicals, such as the chlorinated benzenes and their hydroxylated metabolites (den Besten *et al.*, 1991 and van den Berg, 1990), or the hydroxylated PCBs (Brouwer *et al.*, 1990; Brouwer and van den Berg, 1986; Cheek *et al.*, 1999; Lans *et al.*, 1993; Rickenbacher *et al.*, 1986). First, the degree of bromine substitution appeared to play a crucial role in the binding potency, as (bis)phenols with a lesser degree of bromination showed lower or no competitive binding to TTR. These results are consistent with earlier studies performed with chlorinated phenols, showing an increased interaction of higher chlorinated phenols with transthyretin as compared to lower chlorinated phenols (den Besten *et al.*, 1991; van den Berg, 1990). Second, the nature of the halogen substitution also plays a major role in the binding affinity of the compounds to TTR. TBBPA was the most potent competitor in this study (relative potency of 10.6 compared to the natural ligand), whereas TCBPA, with the only structural difference being the bromine atoms replaced by chlorine atoms, competed with T_4-TTR binding with lesser potency than TBBPA. Higher

binding potency of brominated analogues over chlorinated ones was also observed for PBP (relative potency 7.14, this study) as compared to PCP (relative potency of 1.74 [van den Berg, 1990] and 2.50 [den Besten *et al.*, 1991]).

Third, comparison of the relative potencies of TBBPA versus triBBPA versus diBBPA and PBP versus 2,4,6-TBP versus 2,4-DBP indicates that hydroxylation at the *para* position with one but preferably two adjacent halogen substituents, which is proposed to be the prerequisite for binding of hydroxylated PCBs to human transthyretin (Lans *et al.*, 1993), is also an essential requirement in the binding of the brominated (bis)phenols to TTR. It is hypothesized that these lateral (3,3',5,5') halogens can occupy the binding pockets of TTR normally occupied by the diiodophenolic ring of the thyroxine molecule, as has been shown for 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (Lans, 1995) and proposed for 3,3',4,4',5,5'-hexachlorobiphenyl (Rickenbacher *et al.*, 1986). On the contrary, hydroxylation is not always a prerequisite for binding, as several parent PCBs have also been shown to interact with human TTR (Chauhan *et al.*, 1998; Cheek *et al.*, 1999; McKinney and Waller, 1994; Meerts, unpublished results; Rickenbacher *et al.*, 1986). This is further substantiated by earlier findings on the existence of different binding modes of T_4 to TTR, e.g., a forward mode with the phenolic ring pointing towards the center in the TTR binding site, and a reversed mode with the phenolic ring positioned towards the mouth of the channel entrance (De la Paz *et al.*, 1992). In addition, our recent observations based on X-ray crystallography data on organohalogen-TTR complexes showed that the hydroxy group in PBP and TBP was not essential for binding to TTR (Ghosh *et al.*, 2000). The mode of binding of these latter compounds to TTR differs from the binding of other organohalogen compounds identified so far and will be described in detail elsewhere (Ghosh *et al.*, 2000). This different binding may explain the similar potency of, e.g., the single-ring structure PBP and the much larger double-ring structure TBBPA.

Of the 17 PBDEs examined in this study, none of the parent compounds competed with T_4-TTR binding. In this case, metabolic conversion is most likely essential for the capability of PBDEs to displace ^{125}I-T_4 from TTR. The potency of a PBDE to compete with T_4 on TTR appeared to be both congener- and metabolic enzyme-specific. CYP2B-enriched liver microsomes were able to catalyze the formation of PBDE metabolites that showed T_4-TTR competition binding potency. Almost none of the higher brominated diphenyl ethers were capable of displacing T_4 from TTR after microsomal incubation. Two explanations are possible for this observation, i.e., higher brominated diphenyl ethers were not metabolized by the differently enriched microsomal preparations, or the metabolites formed were not able to compete with T_4 for binding to TTR. Further studies will be focused on the elucidation of the chemical identity of these PBDE metabolites. The results with the synthetic OH-PBDEs resembling the thyroid hormones are in good agreement with the competitive binding with T_4 on TTR and other structural analogues (Andrea *et al.*, 1980). However, the small difference in binding affinities between the T_4-like and T_3-like OH-PBDEs (the binding affinity of the T_3-like OH-BDE is a factor 1.1 smaller compared to the T_4-like) cannot be explained at the moment. Comparing the binding affinities of the natural ligands T_4 and T_3 (3.5×10^7 and 3.2×10^6 M^{-1} respectively, i.e., a factor 11 difference [Andrea *et al.*, 1980]), one would expect the affinity of the T_3-like OH-BDE to differ from the T_4-like in the same range. Further studies are necessary to determine the binding of these brominated thyroid hormone analogues to TTR in more detail.

The interaction of brominated flame retardants with transthyretin may indicate interaction with other thyroid hormone-binding proteins such as enzymes involved in thyroid hormone metabolism. Hydroxylated PCBs with high affinity for TTR have been shown to interact with iodothyronine 5'-deiodinase (Adams *et al.*, 1990; Lans, 1995; Rickenbacher *et al.*, 1989) and iodothyronine sulfotransferase (Schuur *et al.*, 1998). However, the interaction of hydroxylated PCBs with thyroxine-binding globulin (TBG), the major thyroid hormone transport protein in humans, is very rare, and affinities are 100-fold lower than T_4 (Cheek *et al.*, 1999; Lans *et al.*, 1994). This may indicate that the impact of compounds binding to transthyretin is lower in humans and nonhuman primates that possess TBG as the major thyroxin carrier. However, the binding of OH-PCBs/OH-PBDEs to TTR may be involved in facilitated transfer of these compounds across the placenta and the blood-brain barrier, leading to relatively high levels in the fetus, and especially the fetal brain. Morse *et al.* (1996a) showed a strong accumulation of the PCB metabolite 4-hydroxy-2,3,3',4',5-pentaCB (4-OH-CB107) in plasma and forebrain of fetuses 20 days of age after exposure of the dams to the commercial PCB-mixture Aroclor 1254 from gestation days 10 to 16. This accumulation could be explained by competition between the 4-OH-CB107 and T_4 for TTR binding, leading to a selective and facilitated transport of the metabolite over the placenta to the fetal compartment. The accompanying reduction in plasma T_4 levels could be caused either by binding of the 4-OH-CB107 to TTR *in vivo* and/or amplified biliary excretion of T_4 due to induction of UDP glucuronosyltransferase (UDPGT) by Aroclor 1254 (Barter and Klaassen, 1992; Morse *et al.*, 1996a; van Birgelen *et al.* 1995). However, exposure of pregnant rats to the 4-OH-CB107 alone resulted in decreased T_4 levels in their fetuses without induction of UDP glucuronosyltransferase (*Chapter 5*), indicating that binding of a compound to TTR *in vivo* can result in lowered plasma levels of T_4 in the rat.

Surprisingly, TBBPA, which showed a high T_4-TTR competitive interaction *in vitro* (this *Chapter*), showed no effects on thyroid hormone levels in fetuses 20 days of age after oral exposure of pregnant rats to 5 mg/kg body weight per day from gestation days 10 to 16 (Meerts *et al.*, 1999). This may be explained by the poor absorption of TBBPA from the gastrointestinal tract in rats and its subsequently high fecal elimination after oral exposure (Meerts *et al.*, 1999; IPCS, 1994), or its fast metabolism, especially to a monoglucuronide, which is excreted in the bile (Larsen *et al.*, 1998).

Reduced serum total and free T_4 levels were also reported in mice and rats treated with the commercial mixture Bromkal 70 (containing about 40% of tetraBDE) and the pure congener 2,2',4,4'-tetraBDE (dosage of 250 mg [= 515 μmol]/kg body weight, Darnerud and Sinjari, 1996). The mechanism of this reduction was not investigated, but these results demonstrate that thyroid hormone homeostasis might also be a sensitive target of PBDEs (or metabolites) *in vivo*. In our study, 2,2',4,4'-tetraBDE itself was not able to bind to TTR *in vitro*. Metabolic conversion of 10 μM 2,2',4,4'-tetraBDE with CYP2B-induced microsomes gave rise to metabolites that competed with T_4 for binding to TTR by more than 60%. The concentration of these metabolites could maximally be 250 nM (with 100% conversion; see Materials and Methods section), but HPLC analysis revealed that only 10% of the total BDE-47 was metabolized (Meerts *et al.*, unpublished results). Obtaining quantitative information about the potency of the formed metabolites is not possible, but our results strongly suggest that hydroxylated PBDEs are able to compete with thyroxine for TTR binding *in vitro*.

In conclusion, some brominated (bis)phenols and hydroxylated PBDEs were found to interact with human transthyretin *in vitro* with high affinity. The structural requirements of the brominated compounds were similar to those observed for the chlorinated compounds studied so far and also for the natural ligand itself. The resemblance between these brominated phenolic compounds and hydroxylated PCBs is striking with respect to TTR interaction. This suggests that at least some components of these classes of brominated flame retardants may also interfere in the thyroid hormone system *in vivo* and may cause possible adverse health effects similar to PCBs. Further studies are aimed at investigating the impact of the findings presented in this paper on the *in vivo* situation.

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CHAPTER 3

In vitro estrogenicity of polybrominated diphenyl ethers (PBDEs), hydroxylated PBDEs and polybrominated bisphenol A compounds

Abstract

Polybrominated diphenyl ethers (PBDEs) are used in large quantities as additive flame retardants in plastics and textile materials. PBDEs are persistent compounds and have been detected in wildlife and in human adipose tissue and plasma samples. In this study, we investigated the (anti)estrogenic potencies of several PBDE congeners, three hydroxylated PBDEs (OH-PBDEs), and differently brominated bisphenol A compounds in three different cell line assays based on estrogen receptor (ER)-dependent luciferase reporter gene expression. In human T47D breast cancer cells stably transfected with an estrogen-responsive luciferase reporter gene construct (pEREtata-Luc), 11 PBDEs showed estrogenic potencies, with concentrations leading to 50% induction (EC_{50}) varying from 2.5 to 7.3 μM. The luciferase induction of the most potent OH-PBDE [2-bromo-4-(2,4,6-tribromophenoxy)phenol] exceeded that of estradiol (E_2), though at concentrations 50,000 times higher. As expected, brominated bisphenol A compounds with the lowest degree of bromination showed highest estrogenic potencies (EC_{50} values of 0.5 μM for 3-monobromobisphenol A). In an ERα-specific, stably transfected human embryonic kidney cell line (293-ERα-Luc), the OH-PBDE 4-(2,4,6-tribromophenoxy)phenol was a highly potent estrogen with an EC_{50} < 0.1 μM and a maximum 35- to 40-fold induction, which was similar to E_2. In an analogous ERβ-specific 293-ERβs-Luc cell line, the agonistic potency of the 4-(2,4,6-tribromophenoxy)phenol was much lower (maximum 50% induction compared to E_2), but EC_{50}-values were comparable. These results indicate that several pure PBDE congeners, but especially OH-PBDEs and brominated bisphenol A-analogues, are agonists of both ERα and ERβ receptors, thus stimulating ER-mediated luciferase induction *in vitro*. These data also suggest that *in vivo* metabolism of PBDEs may produce more potent pseudo-estrogens.

Based on: Meerts I.A.T.M., Letcher R.J., Hoving S., Marsh G., Bergman Å., Lemmen J.G., van der Burg B., and Brouwer A. *Env. Health Perspect.* **109**, 399-407 (2001).

Introduction

Polybrominated diphenyl ethers (PBDEs) are widely used as additive flame retardants in many different polymers, resins and substrates at concentrations ranging from 5 to 30% (IPCS, 1994). Because of the widespread production and use of PBDEs, their high binding affinity to particles, and their lipophilic characteristics, several PBDE congeners bioconcentrate and bioaccumulate in the environment in a manner similar to the structurally related polychlorinated biphenyls (PCBs) (IPCS, 1994; De Boer *et al.*, 1999; Pijnenburg *et al.*, 1995). PBDEs have been detected in various biotic samples such as birds, seals, whales, and even in human blood, adipose tissue and breast milk (De Boer *et al.*, 1998; Haglund *et al.*, 1997; Jansson *et al.*, 1987; Lindström *et al.*, 1999; Meironyté *et al.*, 1999; Sjödin *et al.*, 1999 and Stanley *et al.*, 1991). The congeners 2,2',4,4'-tetraBDE (BDE-47), 2,2',4,4',5-pentaBDE (BDE-99), and 2,2',4,4',6-pentaBDE (BDE-100) are generally the dominant congeners found in wildlife and humans. The relevance of PBDEs as environmental contaminants has been demonstrated by their accumulation in human breast milk, where concentrations in Swedish women have increased over the last 2 decades from 0.07 ng/g lipid weight in 1972 to 4.02 ng/g lipid weight in 1998 (Meironyté *et al.*, 1999). Although PCB concentrations in wildlife are still higher than PBDE concentrations, they are declining over the same time period.

The most sensitive end points of PBDE toxicity *in vivo* are effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production [i.e. lowering of free and total thyroxine (T_4) concentrations] in rats and mice (Darnerud *et al.*, 1996; Fowles *et al.*, 1994). Consistent with these findings is the recent observation that several pure PBDE-congeners were able to displace T_4 from transthyretin (TTR; a plasma transport protein of thyroid hormones) *in vitro*, after metabolic conversion to hitherto unidentified metabolites (Meerts *et al.*, 2000, *Chapter 2*). These phenomena have also been observed for other organohalogen compounds such as PCBs and their hydroxylated metabolites (Brucker-Davis, 1998; Brouwer *et al.*, 1998 and references therein).

Another property that PBDEs share with PCBs and the polybrominated biphenyls (PBBs) is the dioxin-like, Ah receptor-mediated induction of cytochrome P450 1A1 and 1A2 *in vitro* (Hanberg *et al.*, 1991) and in *vivo* (Von Meyerinck *et al.*, 1990). Recently we demonstrated by means of an Ah receptor-mediated, chemically activated luciferase expression cell line (the Ah-CALUX-assay) (Aarts *et al.*, 1995; Garrison *et al.*, 1996 and Murk *et al.*, 1996) that several pure di- to hepta-brominated PBDE congeners were able to act via this Ah receptor pathway *in vitro* as agonists and antagonists in a congener specific manner (Meerts *et al.*, 1998). For example 2,3,4,4',5,6-hexaBDE (BDE-166) and 2,3,3',4,4',5,6-heptaBDE (BDE-190) were relatively strong Ah receptor agonists with potencies comparable to the mono-*ortho* 2,3,3',4,4'-pentaCB (CB-105) and 2,3',4,4',5-pentaCB (CB-118) (Sanderson *et al.*, 1996).

Some studies have indicated that hydroxylated PBDEs (OH-PBDEs) are of potential environmental importance. In liver microsomes of rats, several PBDE congeners were biotransformed to metabolites (*Chapter 2*). Örn and Klasson-Wehler (1998) demonstrated that 2,2',4,4'-tetraBDE (BDE-47) is biotransformed to OH-PBDEs in rats and mice. 3,5-Dibromo-2-(2,4-dibromophenoxy)phenol is a hydroxy-BDE that has been identified in blood plasma of Baltic salmon (Asplund *et al.*, 1999) at levels similar to those of the major PBDE congeners. Information on the endocrine activity of hydroxylated PBDEs is presently limited to the ability of several OH-PBDEs to bind competitively to the thyroid hormone receptor (Marsh *et al.*, 1998) and to TTR (Meerts *et al.*,2000; *Chapter 2*).

Studies showing that many industrial chemicals are weakly estrogenic compared to natural estrogens (Zava *et al.*, 1997; Jobling *et al.*, 1995 and Soto *et al.*, 1995) have raised concern about their safety. For example, *o,p'*-DDT, bisphenol A, nonylphenol, and various phthalates possess estrogenic activity (Jobling *et al.*, 1995). The presumption is that these xenoestrogens may disrupt normal endocrine function, which can lead to reproductive failure and cancer of estrogen-sensitive tissues in humans and wildlife (Colborn *et al.*, 1993). Anti-estrogenic activity by anthropogenic compounds has received less attention (Navas and Segner, 1998). Although the inhibition of hormone action and the resulting toxicological consequences have not been demonstrated conclusively, anti-estrogenic action could critically affect sensitive reproductive and developmental processes as well (Navas and Segner, 1998). To date there have been no reports investigating the (anti-)estrogenic activities of PBDEs and OH-PBDEs.

The aim of this study was to determine the (anti-)estrogenicity of 17 PBDE congeners. We also examined three hydroxylated PBDEs that have halogen substitution patterns similar to those of thyroid hormones. The (anti-)estrogenic activity of these compounds was tested *in vitro*, using an estrogen-responsive luciferase reporter cell line (T47D.Luc) (Legler *et al.*, 1999). We compared the structure-activity relationships for (anti-)estrogenicity of PBDE and OH-PBDE congeners with numerous other brominated flame retardants, such as differently brominated bisphenol A compounds. We also tested the most potent PBDEs and OH-PBDEs observed in T47D.Luc cells for estrogen receptor specificity using 293 human embryonic kidney cells stably transfected with recombinant human estrogen receptor (ERα or ERβs) cDNA and the luciferase reporter gene construct (Seinen *et al.*, 1999; Lemmen, unpublished data; Kuiper *et al.*, 1998).

Materials and Methods

Chemicals

The 17 PBDE congeners (> 98% pure; Figure 3.1, Table 3.1) were synthesized as described earlier (Marsh *et al.*, 1999; Örn *et al.*, 1996). Three OH-PBDEs, 4-(2,4,6-tribromophenoxy)phenol (T_2-like OH-BDE), 2-bromo-4-(2,4,6-tribromophenoxy)phenol (T_3-like OH-BDE) and 2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol (T_4-like OH-BDE) (Figure 3.1) were synthesized as described by Marsh *et al.* (1998) and were at least 99% pure. We use the abbreviations for these OH-PBDEs (T_2-like-, T_3-like-, T_4-like OH-BDE) according to their resemblance in halogen substitution patterns to the thyroid hormones 3,5-diiodothyronine (3,5-T_2), 3,3',5-triiodothyronine (T_3) and 3,3',5,5'-tetraiodothyronine (T_4). The core structure of PBDEs and the structures of the OH-PBDEs used in this study are shown

in Figure 3.1, including the structure of the analogue 4-phenoxyphenol. The numbering system for individual PBDE congeners is based on the numbering system applied to PCBs (Ballschmiter *et al.*, 1980).

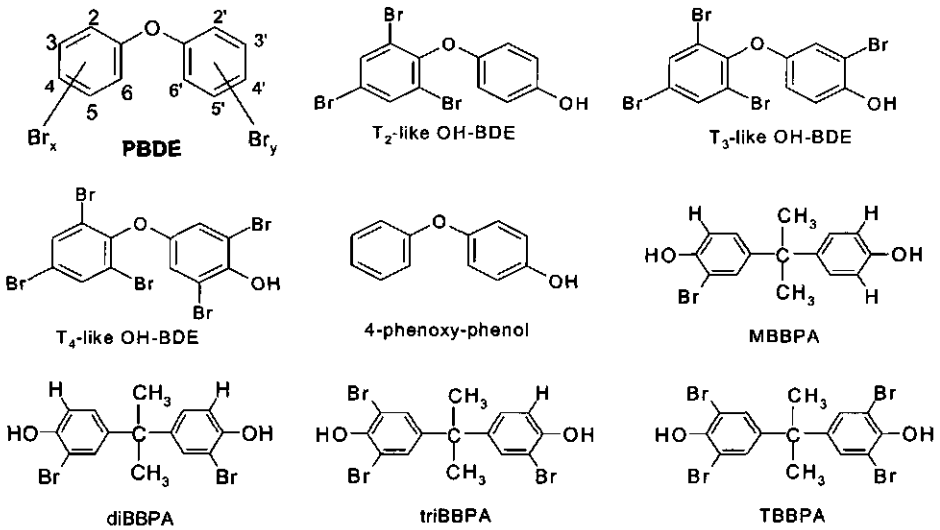

Figure 3.1. Structure of PBDEs, the three hydroxylated PBDEs, 4-phenoxyphenol and the differently brominated bisphenol A analogues.

4-Phenoxyphenol and bisphenol A were obtained from Aldrich Chemical Company (Bornem, Belgium). 17β-Estradiol (E_2, 99%) and ethanol (100%, pro analysis) were purchased from Sigma Chemical Company (St Louis, MO, USA). ICI 182,780 was a gift from A. Wakeling, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK). 3-Monobromobisphenol A (MBBPA; 96.5% pure, with 3.5% 3,3'-dibromobisphenol A), 3,3'-dibromobisphenol A (diBBPA; 99.4% pure, with 0.6% 3,3',5-tribromobisphenol A) and 3,3',5-tribromobisphenol A (triBBPA; 100% pure) were synthesized by bromination of bisphenol A using bromine in acetic acid at room temperature. The test chemicals and E_2 were dissolved in ethanol or dimethyl sulfoxide (DMSO; 99.9% pure, Janssen Chimica, Geel, Belgium) for use in the *in vitro* assays.

Cell culture

We used the human T47D breast cancer cell line stably transfected with an estrogen-responsive luciferase reporter gene construct (pEREtata-Luc) (Legler *et al.*, 1999) to study the *in vitro* (anti)estrogenic activity of PBDEs and OH-PBDEs. The T47D.Luc cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's (DMEM) medium and Ham's F12 (DF) medium (Gibco Brl, Life Technologies, Breda, The Netherlands) supplemented with sodium bicarbonate, nonessential amino acids, sodium pyruvate, and 7.5% fetal calf serum (heat-inactivated) at 37°C and 7.5% CO_2.

The preparation of the stably transfected 293-Luc cell lines (ERα and ERβs) has been described in detail elsewhere (Seinen *et al.*, 1999). Briefly, human 293 embryonal kidney (HEK) cells (ATCC, American Type Culture Collection, Rockville, MD, USA) were stably transfected with the pEREtata-Luc construct (Legler *et al.*, 1999; Seinen *et al.*, 1999) cotransfected with an antibiotic resistance gene. This cell line was subsequently transfected with a recombinant human estrogen receptor (ERα or a short form of ERβ, ERβs) cDNA and a different antibiotic resistance gene. The 293-ERα- and 293-ERβs-Luc cell lines were cultured in a 1:1 mixture of DMEM and DF medium supplemented with 7.5% fetal calf serum (heat-inactivated) at 37°C and 7.5% CO_2.

ER-CALUX assay

We performed the T47D.Luc-based assay as described previously (Legler *et al.*, 1999). The cells were trypsinized, resuspended in assay medium, and seeded in 96 well plates (Packard, Meriden, CT, USA) at a density of 5,000 cells per well in 100 µl. The assay medium consisted of phenol red-free DF and fetal calf serum treated with 5% dextran-coated charcoal (DCC-FCS). DCC-FCS was prepared as described by Horwitz and McGuire (1978). After 24 h, when wells were approximately 50% confluent, the assay medium was renewed. After another 24 h, the assay medium was replaced by incubation medium (for preparation, see below), containing DMSO or ethanol stock solutions of the test compounds or estradiol. Solvent concentrations did not exceed 0.1%. The incubation medium was removed after an incubation of 24 h at 37°C in an atmosphere of 7.5% CO_2. Cells were washed twice with 100 µl phosphate buffered saline (PBS) and subsequently lysed in 30 µl low salt (LS) buffer containing 10 mM Tris (pH 7.8), 2 mM dithiothreitol (DTT), and 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid. After 10 minutes of incubation on ice, the 96 well plates were frozen at -80°C for a minimum of 30 min and maximum of 1 day to lyse the cells. The plates were thawed on ice and shaken for 5 min at room temperature. We measured luciferase activity in a luminometer (Labsystems Luminoscan RS, Breda, The Netherlands) with automatic injection of 100 µl flash mix (pH 7.8) per well containing 470 µM luciferin, 20 mM trycine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 5 mM ATP and 2 mM DTT (pH 7.8).

293-ERα- and 293-ERβs-Luc assay

The 293-ERα- and 293-ERβs-Luc-based assays were performed similarly to the ER-CALUX assay and have been described previously (Seinen *et al.*, 1999; Kuiper *et al.*, 1998). Briefly, cells were trypsinized and resuspended in assay medium composed of phenol red-free DF containing 30 nM selenite, 10 µg/ml transferin, and 0.2% BSA supplemented with 5% DCC-FCS. The cells were seeded in 96-well plates at a density of 15,000 cells per well in 200 µl assay medium. After 48 hr the cells were 50-60% confluent, and the assay medium was replaced by incubation medium (i.e., containing a 1,000-fold dilution of test compounds) as described for the ER-CALUX assay. After an incubation of 24 hr at 37°C in an atmosphere of 7.5% CO_2, the plates were transferred to ice and the medium was removed by suction. Luciferase production was assayed as described above for the ER-CALUX assay.

Exposure of cells

Before the T47D.Luc cell incubations, the PBDE and OH-PBDE stock solutions (prepared in DMSO) and the brominated bisphenol compounds (prepared in ethanol) were diluted 1,000-fold in assay medium in a 48 well plate (to obtain a solvent-concentration of 0.1% v/v) and thoroughly shaken, and 100 µl was added to the cells in 96 well plates. The nominal concentration of the toxicants in the medium were 0.05, 0.1, 0.5, 1.0 and 5 µM, and for potent compounds concentrations of 2.5 and 10 µM were also included. For each experiment, we included a complete E_2 standard curve (1-100 pM, seven different concentrations in total). In addition, we tested three calibration points (0, 10 and 30 pM E_2) on every 96-well plate within an experiment.

For the 293-ERα- and 293-ERβs-Luc assays, the DMSO stock solutions of the tested compounds were diluted 1,000-fold in the appropriate assay medium. The nominal concentrations of the toxicants exposed to the cells were 1.0, 5.0 and 10 µM. For each experiment a complete E_2 standard curve (0.001-10,000 pM in eight different concentrations) was included. For all three ER-CALUX assays, we tested every toxicant concentration in triplicate and repeated each assay at least twice.

Anti-estrogenic effects

We tested the possible anti-estrogenic effects of the compounds in the ER-CALUX assay at the same nominal concentrations as for the estrogenic activity screening. The T47D.Luc cells were coincubated with an E_2 concentration of 10 pM. This E_2 concentration was the approximate EC_{50} for the induction of luciferase activity (Legler *et al.*, 1999). The percentage (v/v) of DMSO present during these anti-estrogenicity incubations was 0.2%. An anti-estrogenic effect in this assay was defined by the capacity of a chemical to inhibit the luciferase activity induced by the approximate EC_{50} concentration of E_2. The percentage inhibition is calculated according to the equation

$$I(\%) = 100 \cdot \left(1 - \frac{L_{test} - L_{control}}{L_{E2} - L_{control}} \right) \qquad (1)$$

where I is the percent inhibition, and L_{test}, $L_{control}$, and L_{E2} are the average luciferase activity of three test wells, three control wells and six wells incubated with 30 pM of E_2, respectively. Using Equation 1, a compound without antagonistic activity will show the same luciferase induction as 10 pM of E_2, [i.e. 63.3 ± 7.5% (see "Results")]. On each plate a positive control of 10 nM of the competitive ER antagonist ICI 182,780 was included in triplicate. ICI 182,780 produces virtually total antagonism of E_2-induced luciferase activity at this concentration [i.e., activity measured is equal to solvent control levels (Legler *et al.*, 1999)].

Cytotoxicity

We measured possible cytotoxic effects of the tested compounds in the bioassays using MTT activity (Denizot and Lang, 1986). To determine cytotoxic effects, we seeded cells and exposed them to the test compounds in the same manner as outlined in their corresponding assay procedures.

Dose-response curves and statistics

Possible dose-response relations were described by the sigmoidal function

$$y = a_0 + a_1/(1 + \exp((a_2 - x)/a_3)) \quad (2)$$

using SlideWrite Plus 4.0 (Advanced Graphics Software, Carlsbad, CA, USA), where y is the induction of luciferase activity compared to controls for estrogenic effects, or inhibition [I (%), Equation (1)] for anti-estrogenic effects, x is the logarithm of the dose, and a_1 is the maximum y-value. We tested the significance of the data fits using one-way analysis of variance at $p < 0.05$.

Results

Cytotoxicity

In the concentration range of 0.01 to 10 μM, none of the incubations of the PBDEs or OH-PBDEs showed any significant effect on MTT activity relative to the solvent control (data not shown). Furthermore, no cytotoxic effect could be observed by microscopic examination in this concentration range. PBDE concentrations could not exceed 10 μM because of solubility problems and slight cytotoxic effects (data not shown).

ER-CALUX assay based on T47D.Luc cells

Estrogenic effects

Seventeen PBDE congeners and 3 OH-PBDEs were tested in the T47D.Luc-based ER-CALUX assay for their estrogenic and/or anti-estrogenic properties. Eleven PBDEs exhibited luciferase induction (Table 3.1) in a dose-dependent manner (Figure 3.2).

The most potent PBDE-congeners [2,2',4,4',6-pentaBDE (BDE-100) > 2,4,4',6-tetraBDE (BDE-75) > 2,2',4,6'-tetraBDE (BDE-51) > 2,4,6-triBDE (BDE-30) > 2,3',4,4',6-pentaBDE (BDE-119)] showed EC_{50} values within a small concentration range of 2.5 to 3.9 μM (Table 3.1). These PBDE agonists were 250,000-390,000 times less potent than the natural ligand, E_2.

Table 3.1. Estrogenic activity of polybrominated diphenyl ethers (PBDEs), hydroxylated PBDEs (HO-PBDEs) and brominated bisphenols in the ER-CALUX assay with T47D.Luc cells.

Compound	Bromine Substitution	LOEC (μM)[a]	Relative Potency (LOEC)[b]	EC_{50} (μM)[c]	Relative Potency (EC_{50})[d]	Max. luciferase induct. (%)[e]
Estradiol		1.0×10^{-6}	–	1.0×10^{-5}	–	100
PBDEs						
BDE-15	4,4'	n.a.*	–	–	–	< 1
BDE-28	2,4,4'	0.5	2.0×10^{-6}	n.a.	–	43 ± 2
BDE-30	2,4,6	0.5	2.0×10^{-6}	3.4	2.9×10^{-6}	114 ± 31
BDE-32	2,4',6	0.05	2.0×10^{-5}	5.1	1.9×10^{-6}	85 ± 13
BDE-47	2,2',4,4'	5.0	2.0×10^{-7}	n.a.	–	6 ± 1
BDE-51	2,2',4,6'	0.5	2.0×10^{-6}	3.1	3.2×10^{-6}	85 ± 18
BDE-71	2,3',4',6	0.5	2.0×10^{-6}	7.3	1.4×10^{-6}	62 ± 8
BDE-75	2,4,4',6	0.5	2.0×10^{-6}	2.9	3.5×10^{-6}	53 ± 10
BDE-77	3,3',4,4'	n.a.	–	–	–	< 1
BDE-85	2,2',3,4,4'	5.0	2.0×10^{-7}	n.a.	–	8 ± 1
BDE-99	2,2',4,4',5	5.0	2.0×10^{-7}	n.a.	–	2 ± 1
BDE-100	2,2',4,4',6	0.05	2.0×10^{-5}	2.5	4.1×10^{-6}	57 ± 10
BDE-119	2,3',4,4',6	0.05	2.0×10^{-5}	3.9	2.6×10^{-6}	25 ± 4
BDE-138	2,2',3,4,4',5'	n.a.	–	–	–	1 ± 1
BDE-153	2,2',4,4',5,5'	n.a.	–	–	–	< 1
BDE-166	2,3,4,4',5,6	n.a.	–	–	–	< 1
BDE-190	2,3,3',4,4',5,6	n.a.	–	–	–	< 1
HO-PBDEs						
4-phenoxy-phenol		0.5	2.0×10^{-6}	1.7	5.8×10^{-6}	195 ± 17
T_2-like OH-BDE		0.05	2.0×10^{-5}	0.1	1.0×10^{-4}	160 ± 11
T_3-like OH-BDE		0.5	2.0×10^{-6}	0.5	2.0×10^{-5}	119 ± 22
T_4-like OH-BDE		n.a.	–	–	–	< 1
(Brominated) bisphenols						
Bisphenol A	0.01	1.0×10^{-4}	0.3	3.3×10^{-5}	200 ± 15	
MBBPA	0.1	1.0×10^{-5}	0.5	2.0×10^{-5}	125 ± 3.1	
DiBBPA	0.1	1.0×10^{-5}	0.4	2.5×10^{-5}	136 ± 1	
TriBBPA	0.5	2.0×10^{-6}	> 10	$< 1.0 \times 10^{-6}$	80 ± 3	
TBBPA	n.a.	–	–	–	< 1	

* Not achieved.

[a] Lowest observed effect concentration; lowest concentration where luciferase activity is detected.

[b] Ratio between dose of compound and estradiol needed to achieve an estrogenic effect ($LOEC_{(E2)}$ / $LOEC_{(compound)}$).

[c] Concentration at which the induction of luciferase activity is 50% of the maximum.

[d] Ratio between EC_{50} of the compound and EC_{50} of estradiol

[e] Per cent luciferase activity induced by the test compound, relative to the maximum luciferase activity of E2 (30 pM). Maximum concentration of the test compounds was 10 μM, with the exception of BDE-47 and BDE-85 (maximum: 5 μM).

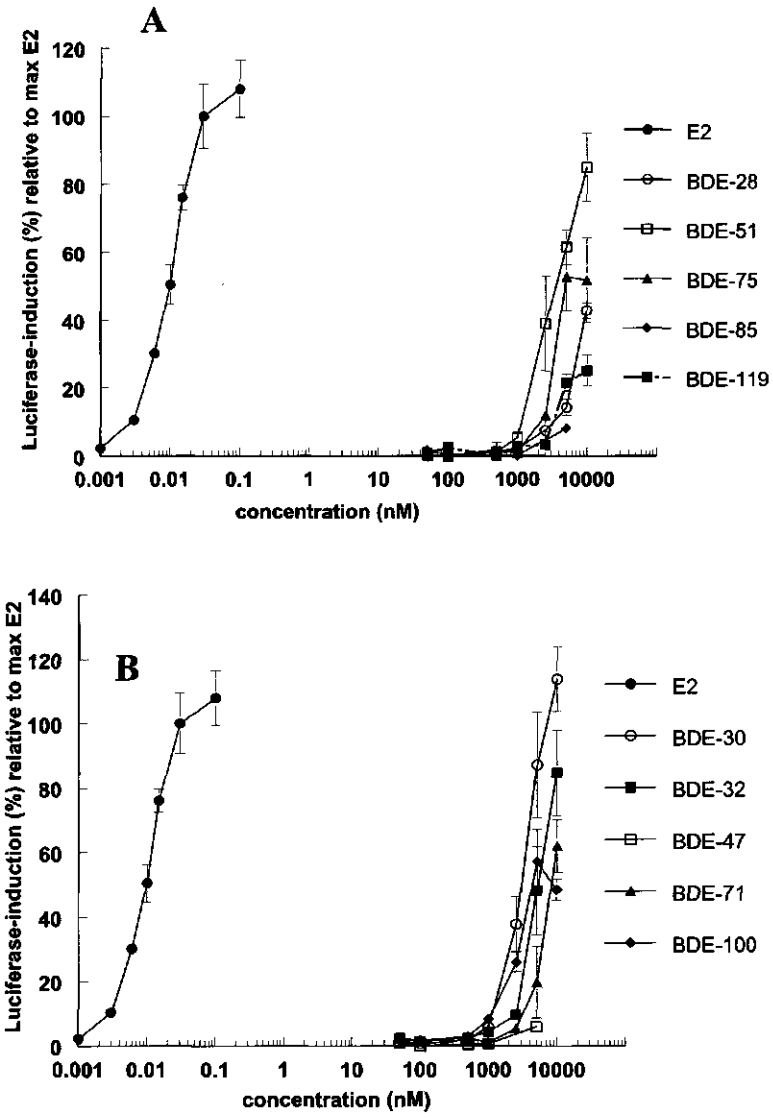

Figure 3.2. Estrogenic activity of PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24 hr exposure to several concentrations of (A) BDE-28, -51, -75, -85, and –119, and (B) BDE-30, -32, -47, -71, and -100. Points are means (n = 3) ± SD (bars) for each concentration.

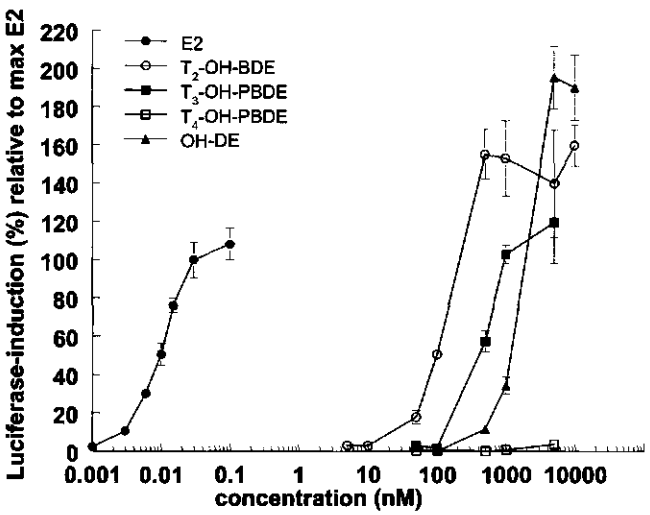

Figure 3.3.
The estrogenic activity of hydroxy-PBDEs in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of T_2-like OH-BDE, T_3-like OH-BDE, T_4-like OH-BDE, and OH-DE (4-phenoxyphenol). Points are means (n=3) ± SD (bars) for each concentration.

The T_4-like OH-BDE compound demonstrated no estrogenic effect up to 10 μM (Figure 3.3). In contrast, the T_3-like OH-BDE and T_2-like OH-BDE showed the highest estrogenic potencies (EC_{50} 0.5 and 0.1 μM, respectively) among all compounds tested in this study (Table 3.1, Figure 3.3). The compound 4-phenoxyphenol was included for comparison because it is structurally analogous to the hydroxylated PBDEs. The T_2-like and T_3-like OH-BDEs induced maximum luciferase activity at 0.5 μM and 1.0 μM respectively, and this maximum luciferase activity (160 ± 11 and 119 ± 22 %) exceeded that of the natural hormone E_2 (Table 3.1).

Of the brominated bisphenols tested, MBBPA and diBBPA showed estrogenic activities comparable to the T_3-and T_2-like OH-BDE, with EC_{50} values of 0.5 and 0.3 μM, respectively (Figure 3.4, Table 3.1). The maximum luciferase activity of bisphenol A, MBBPA, and diBBPA exceeded the maximum activity induced by E_2 (Figure 3.4). Bisphenol A and 4-phenoxyphenol had the highest maximum luciferase activity of 199 ± 15% and 195 ± 17%, respectively, relative to the maximum of E_2 (set at 100%, Figure 3.4). Tetrabromobisphenol A (TBBPA) showed no estrogenic potency within the tested concentrations (Figure 3.4).

Anti-estrogenic effects

The anti-estrogenic potency of PBDEs was determined in the ER-CALUX bioassay by treating T47D.Luc cells with 0.01 to 10 μM concentrations of PBDEs in the presence of 10 pM of E_2. Alone, this E_2 concentration produced a luciferase induction of 63.3 ± 7.5% of the maximum (Table 3.2). At the 10 nM concentration, the ER antagonist ICI 182,780 completely inhibited the luciferase activity induced by 10 pM E_2. Only 2,2',4,4',5,5'-hexaBDE (BDE-153), 2,3,4,4',5,6-hexaBDE (BDE-166), and 2,3,3',4,4',5,6-hepta-BDE (BDE-190), which did not induce luciferase activity alone (up to 10 μM, Table 3.1), reduced E_2-induced luciferase activity (Table 3.2). Moreover, these three PBDE congeners inhibited the E_2-induced activity in a dose-dependent manner (Figure 3.5).

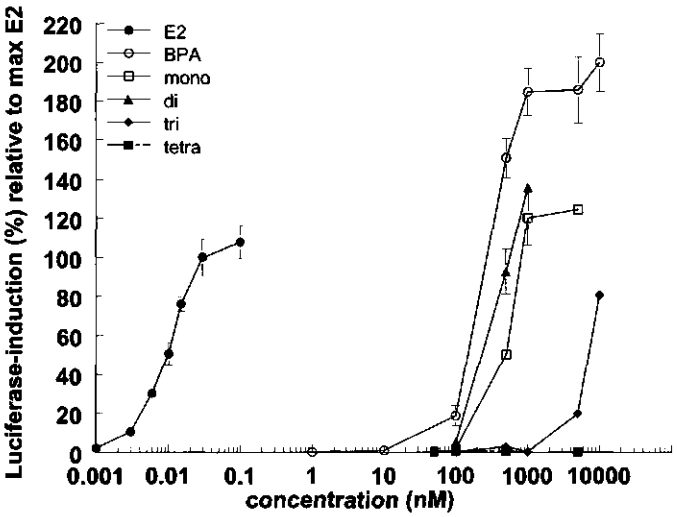

Figure 3.4. Estrogenic activity of differently brominated bisphenols in the T47D.Luc cells. Luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of bisphenol A (BPA), MBBPA (mono), diBBPA (di), triBBPA (tri), and TBBPA (tetra). Points are means (n=3) ± SD (bars) for each concentration.

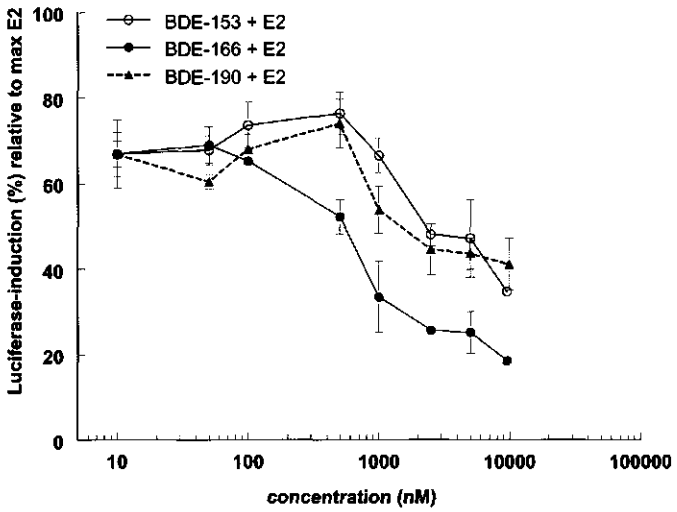

Figure 3.5. Anti-estrogenic activity of PBDEs in the T47D.Luc cells. The luciferase induction (%) relative to the maximum induction by E_2 (30 pM) after 24-hr exposure to several concentrations of BDE-153 (2,2',4,4',5,5'-hexaBDE), BDE-166 (2,3,4,4',5,6-hexaBDE) and BDE-190 (2,3,3',4,4',5,6-heptaBDE), in the presence of 10 pM E_2 (with luciferase induction of 63.3 ± 7.5% of the maximum induction). Points are means (n=3) ± SD (bars) for each concentration.

between ER and THR interactions emphasizes that nonbromination of the phenolic ring is necessary for optimum interaction with the ER, which was also found for OH-PCBs (Korach *et al.*, 1988; Connor *et al.*, 1997). Conversely, like the interaction of the natural, iodine-containing T_2, T_3, and T_4 thyroid hormones with THR and TTR, increasing bromination in adjacent positions on the OH-PBDEs increases THR and TTR binding affinity. The same is true for the brominated bisphenols. The ranking of estrogenic potency in the T47D.Luc cells of the brominated bisphenols was monoBBPA (EC_{50}, 0.5 μM) ~ diBBPA (EC_{50}, 0.3 μM) >> triBBPA (EC_{50} > 10 μM) >>> TBBPA, and was also the reverse order found for interaction with human TTR *in vitro* (*Chapter 2*). The addition of bromine atoms in the *meta* position of the aromatic ring (in diBBPA) had no significant effect on the estrogenic potency. This is in line with results published by Perez *et al.* (1998), where the estrogenicity of 2,2-bis(4-hydroxy-3-methylphenyl)propane (i.e. one methylgroup in the *meta* position of one aromatic ring) in a bioassay with MCF7 human breast cancer cells was not changed compared to bisphenol A. However, the introduction of two bromine atoms in the *meta* position of one aromatic ring drastically decreased the estrogenic potency (triBBPA, this study).

In contrast to the OH-PBDEs, the major OH-PCBs identified in human serum were mostly anti-estrogenic but exhibited low to nondetectable estrogenic activities in several *in vitro* bioassays (Brotons *et al.*, 1995). At concentrations as high as 10 M, several 4-OH-substituted PCBs were not estrogenic toward binding of rat uterine ER. Furthermore, the same OH-PCBs did not induce the proliferation of MCF7 human breast cancer cells, or the luciferase activity of transiently transfected HeLa.Luc cells and MCF7 cells. Unlike the present OH-PBDEs, these OH-PCBs possessed tri- to tetrachlorine substitution on the phenolic ring. In this study, only three of the PBDEs [2,2',4,4',5,5'-hexaBDE (BDE-153), 2,3,4,4',5,6-hexaBDE (BDE-166) and 2,3,3',4,4',5,6-hepta-BDE (BDE-190)] showed anti-estrogenic activities with concentrations resulting into 50% inhibition (IC_{50} values) ranging from 0.8 to 3.1 μM. These PBDEs are likely not metabolized *in situ* because the congeners are hexa- or hepta-bromine substituted, have two *para*-bromines, and have no adjacent or *ortho-meta* brominated carbons. Since the T47D.Luc cells express a functional Ah-receptor, it may be possible that the anti-estrogenicity of these PBDEs is Ah-receptor mediated, as is the case for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and several other anti-estrogens (Safe *et al.*, 1998). BDE-153, -166 and -190 induced the highest maximal luciferase activity in an Ah-receptor CALUX assay based on H4IIE.Luc cells, among the same set of 17 PBDEs (Meerts *et al.*, 1998).

The anti-estrogenicity of Ah receptor ligands is directly correlated to their affinity for the Ah receptor and their CYP1A-inducing potency (Safe *et al.*, 1998). As shown for TCDD-treated MCF7 cells (Krishnan and Safe, 1993), the result is enhanced estrogen catabolism, and lower availability of estrogen to the cell. This correlation between structure-anti-estrogenicity- and structure-CYP1A-inducing potency has been shown for various halogenated aromatics such as TCDD and non-*ortho* PCBs *in vivo* and *in vitro* (Zacharewski *et al.*, 1994; McKinney and Waller, 1994). The exact mechanism of anti-estrogenicity is probably specific to species, cell type, and the estrogen-responsive gene. Other possible cellular mechanisms of Ah receptor-mediated anti-estrogenicity of BDE-153, -166 and -190 may be that the Ah receptor decreases the binding of the ER to the estrogen-responsive element, or the Ah receptor could act as a repressor by inhibiting the binding of other transcription factors (ER) or the disruption of promotor function.

Interestingly, the OH-PBDEs induced luciferase to a higher maximum activity than the maximum induction generated by E_2, though at higher concentrations. This has been shown for several other compounds mimicking the natural estrogen in reporter gene assays. Legler *et al.* (1999) reported this phenomenon for the environmental estrogens genistein, nonylphenol, bisphenol A, *o,p'*-DDT and methoxychlor in the same T47D.luc cells. Routledge and Sumpter (1996) showed that genistein and 4-*tert*-octylphenol induced luciferase activity at a higher level than estradiol in a recombinant yeast strain. The mechanism of this high induction is not yet resolved, but effects on luciferase stability or stimulation of the expression of the receptor or co-activation factors are hypothesized to be involved (Legler *et al.*, 1999).

We detected no striking differences in the relative binding affinities for the tested compounds between ERα or ERβ. However, the agonistic activity compared to E_2 of BDE-30 and BDE-100 was much higher in the 293-ERα- than in the 293-ERβs-Luc cell line (Figure 3.6). Moreover, the agonistic activity of T_2-like OH-BDE, but not 4-phenoxyphenol, was estrogen receptor dependent (Figure 3.6). The induction of luciferase compared to E_2 by T_2-like OH-BDE was much higher in the 293-ERα-Luc assay, whereas the induction of luciferase by 4-phenoxyphenol was not selective to either assay. This would suggest that the presence of a bromine atom adjacent to the phenolic hydroxyl group is a discriminating factor leading to a partial agonistic activity in the 293-ERβs-Luc cell line compared to a full agonistic activity in the 293-ERα-Luc cell line.

In the same two ER-CALUX assays, polycyclic musk compounds were selective to the 293-ERα-Luc but not the 293-ERβs-Luc assay (Seinen *et al.*, 1999). OH-PCBs with chlorine atoms only on the nonphenolic ring were found to bind with purified human ERα and ERβ with at least a 10-fold greater affinity than OH-PCBs with chlorine atoms on the phenolic ring (Kuiper *et al.*, 1998). However, the binding preference was 2-fold greater for the ERβ over the ERα. In the same study, 4-OH-2',4',6'-trichlorobiphenyl and 4-OH-2',3',4',5'-tetrachlorobiphenyl highly induced luciferase activity in transiently transfected 293-ERα-Luc and 293-ERβs-Luc cells, although the transactivation activity was higher in the 293-ERα-Luc cells.

In conclusion, the results from this study clearly demonstrate that several pure PBDE congeners, but especially hydroxylated PBDEs and polybrominated bisphenol A compounds, induce the estrogen receptor signal transduction pathway *in vitro*. The estrogenic potencies of these flame retardants are in the same range as the well-known environmental estrogen bisphenol A. The structure-activity relationships of the PBDEs are in accordance with structure-activity relationships proposed for hydroxylated polychlorinated biphenyls. Further, the agonistic potency *in vitro* of estrogenic PBDEs and OH-PBDEs is preferential toward the ERα relative to ERβ. Because of the high-production volume of these compounds and their accumulation in the environment, further studies on the possible implications of these findings for the *in vivo* situation are necessary.

Acknowledgements

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TTR is a tetrameric molecule formed by four identical polypeptide chains (each of 127 amino acid residues). The subunits of TTR are linked together in such a way, that the three dimensional structure of TTR reveals a compact molecule with a channel running through its center. This channel contains two symmetry-related binding sites of the thyroid hormones. The thyroid hormone T_4 can bind to TTR in different orientations, e.g. a "forward mode" with the phenolic ring pointing towards the center in the TTR binding site, and a "reversed mode" with the phenolic ring positioned towards the mouth of the channel entrance (De la Paz *et al.*, 1992). The forward mode is the most common, because the hydroxyl groups of T_4 bound to TTR form hydrogen bonds within the molecule. This formation of hydrogen bonds was suggested to be an important requirement for strong binding of compounds to TTR, and is observed for most ligands, including e.g. 4,4'-$(OH)_2$-3,3',5,5'-tetrachlorobiphenyl (Lans, 1995). However, examination of the PBP-TTR and the TBP-TTR complexes revealed a new mode of binding (Ghosh *et al.*, 2000). In both complexes, PBP and TBP were bound to TTR exclusively in the reversed mode, which is very rare. The only other example of such exclusive reversed binding has been observed for flufenamic acid (Peterson *et al.*, 1998). Thus, the results from our X-ray crystallography studies with PBP and TBP show that strong binding can also take place in the absence of the hydrogen-bond interaction. This is an important finding, since it may imply that an even larger number of organohalogen compounds have the potential to compete with thyroxine and consequently adversely affect the thyroid hormone system in animals and humans.

In *Chapter 3*, the (anti-)estrogenic potency of the same set of 17 pure PBDE congeners, three synthetic OH-PBDEs and differently brominated bisphenols is described. PBDE congeners with the highest estrogenic activity were 2,2',4,4',6-pentaBDE (BDE-100), 2,4,4',6-tetraBDE (BDE-75) and 2,2',4,6'-tetraBDE (BDE-51). Their relative potencies compared to estradiol were 4.1×10^{-6}, 3.5×10^{-6} and 3.2×10^{-6}, respectively. As a comparison, the estrogenic potency of the well known estrogenic compound bisphenol A was 3.3×10^{-5}. The common structural features of PBDEs with estrogenic activity deduced from these studies resemble the ones observed for OH-PCBs, namely two *ortho* (2,6 substituted)-bromine atoms on one phenyl ring, at least one *para*-bromine atom (preferably on the same phenyl-ring as the *ortho* bromines), and nonbrominated *ortho-meta* or *meta* carbons on the other phenyl ring. In contrast with most OH-PCBs detected in human blood, which were anti-estrogenic, only three of the tested PBDEs showed anti-estrogenic activity, namely 2,2',4,4',5,5'-hexaBDE (BDE-153, IC_{50} = 3.1 μM); 2,3,4,4',5,6-hexaBDE (BDE-166, IC_{50} = 0.8 μM) and 2,3,3',4,4',5,6-heptaBDE (BDE-190, IC_{50} = 1.0 μM). Since these same PBDE congeners also possessed dioxin-like activity as measured using an Ah receptor based reporter gene assay (CALUX) in H4IIE.luc cells (Meerts *et al.*, 1998), it is postulated in *Chapter 3* that the anti-estrogenicity observed for these PBDEs may be Ah receptor mediated.

Comparison between the structural requirements of brominated compounds regarding their thyroidogenic or estrogenic activity reveals some interesting features. The most striking observation is the fact that in case of OH-PBDEs or brominated bisphenols, non-bromination on the phenolic ring is favoring the estrogenic potency of the compounds, whereas increasing adjacent bromination on the phenolic ring favors the thyroidogenic potency. Consequently, as discussed in *Chapter 3,* the ranking

order for OH-PBDEs and brominated bisphenols for estrogenicity was the complete reversed order as observed for thyroidogenicity. In Table 4.1 the ranking order of PBDEs and hydroxylated PBDEs regarding their estrogenicity, thyroidogenicity and dioxin-like activity is summarized. Table 4.2 summarizes the ranking order of brominated (bis)phenols regarding their estrogenicity and thyroidogenicity.

In conclusion, the results of the *in vitro* studies with PBDEs presented in this thesis show that the toxicity profile of PBDEs resembles the profile known for (hydroxy) PCBs, especially concerning their thyroidogenic potencies. An important question is, if these thyroidogenic potencies, e.g. the binding to TTR *in vitro* would have any consequences for the *in vivo* situation. Several studies are described in the literature addressing this question, using single PCB congeners or commercial PCB mixtures which were administered to pregnant rats and mice to examine the effects on the dams and the developing offspring. However, since the parent compounds used in these studies are able to exert adverse effects on their own, it is not possible to discriminate between the effects caused by metabolites that were formed *in vivo* and the effects caused by the parent compounds. This prompted us to investigate the possible adverse effects of *in vivo* exposure to a pure, hydroxylated compound, that was shown to bind to TTR, in more detail. For this purpose, the PCB metabolite 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) was chosen as a model compound, because it was detected in human blood, it was known to bind to TTR *in vitro* with a three times higher potency compared to the natural ligand T_4 (see *Chapter 5*), and because exposure of pregnant rats to Aroclor 1254 resulted in the selective retention of this metabolite in fetal plasma and tissues. As presented in the following Chapters, the presence of hydroxylated PCBs in human plasma that are able to bind to TTR may have toxicological consequences for the developing fetus.

Table 4.1. Summary of the PBDEs and OH-PBDEs with (anti-)estrogenic, thyroidogenic or dioxin-like activity. Compounds are given in decreasing order of potencies, with their corresponding EC_{50} or IC_{50} values.

Estrogenic	**EC_{50} µM**	*Anti-estrogenic*	**IC_{50} µM**	**Thyroido-genic**	**IC_{50} nM**	**AhR-agonist**	**EC_{50} µM**	**AhR-antagonist**	**IC_{50} µM**
PBDEs									
Substitution		*Substitution*				*Substitution*			
2,4,4',6	2.9	2,3,4,4',5,6	0.8	none	–	2,3,3',4,4',5,6	0.8	2,2',3,4,4',5'	0.3
2,2',4,6	3.1	2,3,3',4,4',5,6	1.0			2,3,4,4',5,6	1.4	3,3',4,4'	1.7
2',4,6	3.4	2,2',4,4',5,5'	3.1					2,2',4,4'	3.6
2,3',4,4',6	3.9							4,4'	8.8
2,4',6	5.1								
2,3',4',6	7.3								
OH-PBDEs									
T_2-like	0.1	none	–	T_4-like	57.0	none	–	none	–
T_3-like	0.5			T_3-like	66.0				
				T_2-like	199.2				

Table 4.2. Summary of (brominated) (bis)phenols with (anti-) estrogenic, thyroidogenic or dioxin-like activity. Compounds are given in decreasing order of potencies, with their corresponding EC_{50} or IC_{50} values.

Estrogenic	EC_{50} µM	*Anti-estrogenic*	IC_{50} µM	**Thyroidogenic**	IC_{50} nM
(Brominated) (bis)phenols					
Bisphenol A	0.3	none	–	TBBPA	7.7
DiBBPA	0.4			PBP	11.5
MBBPA	0.5			TBP	67.2
				TCBPA	106.8
				TriBBPA	140.0
				2,4-DiBP	1.4×10^3
OH-PBDEs					
T_2-like	0.1	none	–	T_4-like	57.0
T_3-like	0.5			T_3-like	66.0
				T_2-like	199.2

Abbreviations used: DBBPA = dibromobisphenol A; 2,4-DiBP = 2,4-Dibromophenol; MBBPA = monobromobisphenol A; TBP= tribromophenol; TCBPA = tetrachlorobisphenol A; T_2-like = 4-(2,4,6-tribromophenoxy)phenol; T_3-like = 2-bromo-4-(2,4,6-tribromophenoxyphenol; T_4-like = 2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol).

PART II

In vivo studies

CHAPTER 5

Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat

Abstract

Earlier studies at our laboratory indicated that several hydroxylated polychlorinated biphenyls (OH-PCBs) detected in human blood could specifically inhibit thyroxine (T_4) transport by competitive binding to the thyroid hormone transport protein transthyretin (TTR) *in vitro*. In the present study we investigated the effects of prenatal exposure to 5 mg/kg body weight of [^{14}C]-labelled or unlabelled 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107), one of the major metabolites of PCBs detected in human blood, from gestation days (GD) 10 to 16 on thyroid hormone status and metabolism in pregnant rats and their fetuses at GD17 and GD20. 4-OH-CB107 is a metabolite of both 2,3,3',4,4'-pentachlorobiphenyl (CB-105) and 2,3',4,4',5-pentachlorobiphenyl (CB-118).

We were able to show the accumulation of 4-OH-CB107 in the fetal compartment. The fetal/maternal ratios at GD20 in liver, cerebellum and plasma were 11.0, 2.6 and 1.2, respectively. The [^{14}C]-4-OH-CB107-derived radioactivity in plasma was bound to TTR in both dams and fetuses. Fetal plasma TT_4 and FT_4 levels were significantly decreased at GD17 and GD20 (by 89% and 41% respectively at GD20). Fetal thyroid stimulating hormone levels were increased by 124% at GD20. The T_4 concentrations in fetal forebrain homogenates at GD20 were reduced by 35%, but no effects could be detected on brain T_3 concentrations. The deiodination of T_4 to T_3 was significantly increased in fetal forebrain homogenates at GD17, and unaltered at GD20. In addition, no alterations were observed in maternal and fetal hepatic T_4-UDP-glucuronosyltransferase activity, type I deiodinase activity and EROD activity.

In conclusion, exposure of pregnant rats to 4-OH-CB107 results in the distribution of the compound in the maternal and fetal compartment, which is probably caused by the binding of the PCB metabolite to TTR. Consequently, TT_4 levels in fetal plasma and brain samples were reduced. Despite reductions in fetal brain T_4 levels, the active hormone (T_3) in fetal brains remained unaffected.

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Introduction

Polychlorinated biphenyls (PCBs) are widespread, persistent environmental pollutants which have been reported to cause a variety of toxic effects, including neurotoxicity, developmental toxicity, reproductive toxicity and carcinogenesis (reviewed in Peterson *et al.*, 1993; Safe 1990, 1994; Schantz 1996; Seegal 1996). In recent years it has become evident that exposure to PCBs can also lead to thyroid hormone disturbances in laboratory animals, wildlife and even humans as reviewed by Brouwer *et al.* (1998). Decreased levels of circulating plasma thyroxine (T_4) following PCB exposure have been shown in both adult (Byrne *et al.* 1987; Barter and Klaassen, 1994; Van den Berg *et al.*, 1988) and developing organisms (Collins and Capen 1980; Darnerud *et al.*, 1996; Morse *et al.*, 1993, 1996a; Ness *et al.*, 1993; Seo *et al.* 1995). Plasma thyroid hormone levels can be decreased by xenobiotic compounds by at least three known mechanisms. Firstly, a direct effect of compounds on the thyroid gland can lead to a decreased synthesis of thyroid hormones, which has been reported in rats after exposure to the commercial PCB-mixture Aroclor 1254 (Collins and Capen, 1980). Secondly, the reduction in thyroid hormone levels can be caused by enhanced biliary excretion of T_4 due to the induction of UDP-glucuronosyltransferases (UDP-GT) (Barter and Klaassen, 1992; Bastomsky, 1974; Van Birgelen *et al.*, 1995). The third known mechanism involved in reduced plasma T_4 levels is the observed binding of PCB metabolites to the plasma thyroid hormone transport protein, transthyretin (TTR), thereby displacing the natural ligand T_4 (Brouwer *et al.*, 1986; Darnerud *et al.*, 1996; Morse *et al.*, 1996a; Rickenbacher *et al.*, 1986).

Transthyretin (TTR) is the only thyroid hormone binding plasma protein that is synthesised both in liver and brain. It is suggested to serve a role in mediating the delivery of T_4 across the blood-brain barrier and the maternal to fetal transport through the placenta (Southwell *et al.*, 1993; Schreiber *et al.*, 1995). In addition, TTR plays an essential role in the determination of free T_4 levels in the extracellular compartment of the brain, which is independent of the homeostasis of T_4 in the body (Schreiber *et al.*, 1995). T_4 in the brain is then converted to the active thyroid hormone, triiodothyronine (T_3) by specific deiodinases (type II deiodinase). An increasing number of chemicals have been reported to bind to human TTR *in vitro*. Parent PCB congeners (Chauhan *et al.*, 1998; Cheek *et al.*, 1999; Rickenbacher *et al.*, 1986) but especially hydroxylated metabolites of PCBs, dibenzo-*p*-dioxins and dibenzo-*p*-furans (Lans *et al.*, 1993) showed competitive binding to human TTR. Recently we were able to detect a new class of compounds, the brominated flame retardants (e.g. polybrominated diphenyl ethers, brominated bisphenols), with high *in vitro* T_4-TTR competition binding potency (Meerts *et al.*, 2000; *Chapter 2*). The *in vivo* effects of the high binding affinity of xenobiotics such as hydroxylated polychlorinated biphenyls (OH-PCBs) to TTR is hypothesised to result in (i) a selective retention of these compounds in plasma, (ii) facilitated transport of the metabolites over the placenta to the fetal compartment and (iii) decreased maternal and fetal plasma T_4 levels by competition with the natural ligand T_4 (reviewed by Brouwer *et al.*, 1998). Several studies support this hypothesis. Bergman *et al.* (1994) detected several OH-PCBs, with high *in vitro* T_4-TTR binding potency, in human serum and wildlife samples environmentally exposed to PCBs. Exposure of rats to 3,3',4,4'-tetrachlorobiphenyl resulted in selective retention of hydroxylated metabolites in plasma and caused marked reductions in plasma thyroxine levels (Brouwer *et al.*,

1990) and vitamin A transport (Brouwer and Van den Berg, 1986) via their binding to TTR. In addition, maternal exposure of rats to Aroclor 1254 from gestation days 10 to 16 resulted in selective accumulation of the metabolite 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) in fetal plasma and brain (Morse *et al.*, 1996a) and was accompanied by very low concentrations of T_4 in both tissues. In a comparable study conducted in mice, Darnerud *et al.* (1996) showed a high and selective accumulation of 4-OH-3,3',4',5-tetrachlorobiphenyl in fetal mouse plasma and reductions in thyroid hormone levels after maternal exposure to 3,3',4,4'-tetrachlorobiphenyl. They were able to identify the metabolite in fetal plasma bound to TTR.

In vivo toxicity data on the effects of hydroxylated PCB-congeners on thyroid hormone homeostasis are scarce, since most *in vivo* studies are conducted with parent compounds that can exert effects of their own (e.g. induction of UDP-GT) and undergo metabolism in the exposed animal to different metabolites. Therefore, in the present study we investigated the effects of maternal exposure to the synthesised PCB-metabolite 4-OH-CB107 on maternal and fetal rat thyroid hormone homeostasis. To determine maternal to fetal transfer, we also studied the uptake and distribution of [^{14}C] radiolabelled 4-OH-CB107. We chose this metabolite, because it was one of the major metabolites identified in human blood samples (Bergman *et al.*, 1994), and was shown to accumulate in fetal plasma and brain after maternal exposure to Aroclor 1254 (Morse *et al.*, 1996a). Furthermore, 4-OH-CB107 was shown to be a metabolite, formed via a 1,2-shift of a chlorine atom, of 2,3,3',4,4'-pentachlorobiphenyl (CB-105) and of 2,3',4,4',5-pentachlorobiphenyl (CB-118) (Sjödin *et al.*, 1998). Both PCB congeners are present in adipose tissue of humans and wildlife and can thus slowly be biotransformed to the 4-OH-CB107 that is retained in the blood. We especially focused on testing the hypothesis that binding of a PCB metabolite to transthyretin *in vivo* would lead to facilitated transfer of the compound to the fetal compartment resulting in decreased thyroid hormone levels in fetal plasma and brain.

Animals, Materials and Methods

Chemicals

4-Hydroxy-2,3,3',4',5-pentachloro-[^{14}C]biphenyl (specific activity: 15.6 mCi/mmol) was prepared from 3,4-dichloroiodo-[^{14}C]benzene, prepared from 3,4-dichloro-[^{14}C]aniline after this compound had been methylated with diazomethane and reacted with iodine, and 2,3,6-trichloro-4-iodoanisol via an Ullman reaction (Bergman *et al.*, 1990). 4-Hydroxy-2,3,3',4',5-pentachloro-[^{14}C]biphenyl was isolated in a chemical and radiochemical purity > 98%. Unlabelled 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) was synthesised as described by Bergman *et al.* (1995). Isopropanol, bovine serum albumin, sucrose, Tris, hydrogen peroxide (H_2O_2), potassium hydroxide, Triton X-100, diisopropyl ether, dithiotreitol, methanol (all solvents were analytical grade) were purchased from Merck Chemical Company (Darmstadt, Germany). Human prealbumin (transthyretin, TTR, 98% pure) was obtained from Sigma Chemical Company, St. Louis, MO, USA. [^{125}I]-L-3',5'-Thyroxine (spec. act. 46 μCi/μg) was from Orange Medical (Tilburg, The Netherlands).

Animals

All experimental procedures involving animals were approved by the Animal Welfare Committee of the Wageningen University. Wistar WU rats (60 females, 30 males; 14 weeks old) were purchased from Charles River (Sulzfeld, Germany) and allowed to acclimatise for three weeks. Throughout the experiment, animals were kept in an artificial 12 h:12 h light-dark cycle with lights on at 06:00 h. Room temperature was maintained at 21 ± 2°C and humidity at 50 ± 10%. Animals were provided rat chow (Hope Farms, Woerden, the Netherlands) and tap water *ad libitum*.

After the acclimatisation period two females were placed in a cage with one male overnight from 17:00 to 8:00 hr. Copulation was examined each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, this day was designated as day 0 of gestation (GD0) and females were housed individually. Body weight of the dams was measured throughout gestation. On day 10 of gestation the pregnant rats were divided randomly into the different treatment groups and transferred to a macrolon, stainless steel cage to facilitate the collection of faeces.

Study on uptake and distribution of [^{14}C]-labelled 4-OH-CB107 in dams and fetuses.

For investigating the uptake and distribution, six pregnant rats received a daily oral dose of 2.3 μCi [^{14}C]-labelled 4-OH-CB107 per kg body weight diluted with unlabelled 4-OH-CB107 for a total exposure dose of 14.6 μmol (5 mg) 4-OH-CB107 per kg bw per day from gestation days 10 to 16. The metabolite was dissolved in corn oil, 5 mg/2 ml. Faeces and urine were collected daily. On GD17 and GD20, three dams per time point were sacrificed under ether anaesthesia and maternal blood was collected via the *vena cava* in heparinized tubes. Maternal kidneys, liver, adrenals, pancreas, lungs, thymus, forebrain, cerebellum, brown adipose tissue, skeletal muscle and abdominal fat were collected for radioactivity analyses. Individual placental/fetal units were carefully removed from the uterus. Fetuses were separated from the placenta, blotted dry with tissue paper and weighed. Fetal trunk blood, obtained by decapitation, was collected in heparinized tubes, pooled per litter and stored on ice until plasma was prepared for thyroid hormone analysis and radioactivity determinations. From 17 days old fetuses, livers and brains (separated in forebrain and cerebellum) were collected and pooled per litter. From 20 days old fetuses, lungs and kidneys were additionally collected and pooled per litter. Organs and placentas were rinsed with 0.9% sodium chloride, blotted dry with tissue paper, weighed and stored at –80°C. Carcasses were stored at –20°C. Cages were rinsed with 200 ml Triton X-100 at the end of the experiment to determine losses of radioactivity. Maternal and fetal plasma, liver and brain samples were also used in biochemical assays described below (n = 3 per time point).

Study on biochemical effects of 4-OH-CB107 in dams and fetuses

In a parallel experiment, pregnant rats received a daily oral dose of 0 or 5 mg 4-OH-CB107 per kg body weight dissolved in corn oil (2 ml/kg body weight) from GD10 to GD16. On GD17 and GD20, four dams per time point and exposure were sacrificed under ether anaesthesia and maternal blood was collected via the vena cava in heparinized tubes. Fetuses were removed and weighed. Fetal trunk blood was collected in heparinized tubes and pooled per litter. Fetal liver and thymus were collected, weighed, frozen on dry ice and pooled per litter. Fetal brains were removed, separated into forebrain and cerebellum, and frozen on dry ice. One fetal forebrain was saved separately for thyroid hormone analysis, the remaining forebrains and cerebella were pooled per litter for analysis of thyroid hormone metabolism and stored at –80°C. From the dams, liver, brain, thymus and plasma were isolated, frozen in liquid nitrogen and stored at –80°C until analysis.

Tissue radioactivity concentrations

Approximately 60 to 100 mg of tissues or tissue homogenates and 25-50 µl of the plasma samples were dissolved in 1 ml Soluene-350 (Packard, St. Louis, MO, USA) in glass scintillation vials. Samples were bleached with 0.5 ml 30% H_2O_2, and total radioactivity was measured two days later with 20 ml Hionic Fluor scintillation fluid (Packard) in a Packard 1600 liquid scintillation counter (LSC). Faecal samples were homogenised with a mortar under liquid nitrogen. Aliquots (± 50 mg) of faeces homogenates were exactly weighed and solubilized with 1 ml Soluene-350 at 50°C during 1-2 hours in closed glass scintillation vials. After addition of 0.5 ml isopropanol samples were incubated at 50°C for another 2 hours. Samples were bleached by the addition of 0.6 ml 30% H_2O_2, and total radioactivity was measured two days later with 20 ml Hionic Fluor by LSC. The carcasses of dams and fetuses were dissolved in 700 ml (dams) or 200 ml (fetuses) 1.5 M potassium hydroxide containing 20% ethanol (v/v). After homogenisation using an Ultra Turrax 0.5 ml aliquots (in total n=10) were bleached with 0.6 ml 30% H_2O_2 and total radioactivity was measured two days later with 20 ml Hionic Fluor. The efficiency of counting was determined by quenching correction curves for the various additions and scintillation fluids. In order to estimate total radioactivity concentrations in plasma and skeletal muscle, the total weight of plasma and skeletal muscle was set at 4% and 40% of the total body weight, respectively.

Sample processing for biochemical purposes

Livers were thawed on ice and homogenised in ice-cold 0.1 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose (3 ml/g liver) using a Potter tube. The homogenate was centrifuged for 30 minutes at 9,000xg (4°C). The resulting supernatant was centrifuged for 90 minutes at 105,000xg and 4°C. The microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.5). Microsomes were stored in aliquots of 1 ml at –80°C until further analysis.

Maternal and fetal (pooled per litter) forebrains were homogenised in a Potter tube in 8 volumes ice-cold 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mM dithiotreitol and stored at –80°C until further analysis. Protein levels in different tissue fractions were determined using the BioRad Protein reagent (Bradford, 1976).

Thyroid hormone analysis

Plasma total T_4 (TT_4), free T_4 (FT_4) and total T_3 (TT_3) were analysed in duplicate using chemiluminescence kits. Plasma thyroid stimulating hormone (TSH) concentrations were analysed with a specific rat TSH immunoassay. All kits were purchased from Amersham (Amersham, Buckinghamshire, UK).

Brain T_4 and T_3 concentrations were determined by specific RIAs in purified extracts, as described before (Morreale de Escobar *et al.*, 1985). Briefly, maternal and fetal forebrain and cerebellum samples were homogenised in methanol, extracted in chloroform-methanol and back-extracted into an aqueous phase. This aqueous phase was purified through Bio-Rad AG 1x2 resin columns (Bio-Rad Laboratories, Richmond, USA), and the iodothyronines were eluted with 70% acetic acid, which was evaporated to dryness. The iodothyronines were analysed in highly sensitive RIAs in duplicate at two different dilutions. Recovery of the extraction procedure was determined in each homogenate by the addition of tracer amounts of [^{131}I]-T_4 and [^{125}I]-T_3.

Thyroid hormone metabolism

Hepatic microsomal T_4 uridine diphosphoglucuronosyl transferase activity (UDP-GT) was determined as described by Beetstra *et al.* (1991) and Visser *et al* (1993). In short, microsomes (1 mg protein per ml) were incubated for 30 minutes at 37°C with 1 µM T_4 and 50,000 cpm [^{125}I]-T_4, 5 mM uridine 5'-diphosphoglucuronic acid, 3.75 mM $MgCl_2$ and 0.125% (w/v) BSA in 75 mM Tris-HCl-buffer (pH 7.8). The final reaction volume was 0.2 ml. The reaction was stopped by addition of 0.2 ml ice-cold methanol, and after centrifugation 0.2 ml supernatant was mixed with 0.8 ml 0.1 N HCl. The amount of [^{125}I]-T_4 glucuronide was analysed by Sephadex LH-20 chromatography (Rutgers *et al.*, 1989).

Hepatic type I 5'-deiodinase activity (D-I) was measured in duplicate in microsomes as described by Mol and Visser (1985). Briefly, microsomes (25 µg protein/ml) were incubated for 30 minutes at 37°C with 1 µM rT_3 and 100,000 cpm [^{125}I-rT_3] in 0.1 M phosphate buffer (pH 7.4) containing 2 mM EDTA and 5 mM DTT. The final reaction volume was 0.2 ml. The reaction was stopped by addition of 0.75 ml 0.1 M HCl, and the produced [^{125}I] was separated from the reaction mixture by Sephadex LH-20 chromatography according to Rutgers *et al.* (1989). Blanks contained microsomes, inactivated by heating.

Brain type II thyroxine 5'-deiodinase activity (D-II) was analysed as described by Visser *et al.* (1982) with slight modifications. Briefly, brain homogenates (0.8 mg protein/ml) were incubated with 2 nM T_4 and ± 50,000 cpm [^{125}I]-T_4, 500 nM T_3 and 1 mM propyl-2-thiouracil in 0.1 M phosphate buffer pH 7.2 containing 1 mM EDTA and 25 mM DTT in a total volume of 0.2 ml. Incubations were carried out at 37°C for 60 minutes. The reaction was stopped on ice by the addition of 0.1 ml 7% (w/v) BSA, followed by 0.5 ml 10% (w/v) trichloroacetic acid. The tubes were centrifuged at 4000 rpm in an Eppendorf centrifuge for 5 minutes and the amount of radioiodide released was determined in 0.5 ml of the supernatant using Sephadex LH-20 chromatography as described above. Blanks contained brain homogenates, which were inactivated by boiling for 10 minutes.

Ethoxy- and pentoxyresorufin-O-deethylase activity

Ethoxyresorufin-O-deethylase (EROD) activity was measured according to the method of Burke *et al.* (1977) adapted for the use in 96 wells plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Millipore, Etten-Leur, the Netherlands). The reaction was performed with 0.1 mg liver microsomal protein per ml in 0.1 M Tris-HCl (pH 7.8) containing 0.4 µM ethoxyresorufin (ER), 1 mg/ml BSA and 0.1 mM NADPH in a total volume of 0.2 ml. The reaction mixtures were preincubated at 37°C for 2 minutes, and the reaction was started by the addition of NADPH. Reactions were stopped after 10 minutes by adding 50 µl 1 M NaOH. The formation of resorufin was detected fluorimetrically (excitation 530 nm, emission 590 nm) and compared with a calibration curve (0-150 nM resorufin). Incubations were carried out in duplicate and results were corrected for blank microsomal incubations without NADPH.

Pentoxyresorufin-O-deethylase (PROD) activity was measured following the same procedure as described above for EROD, with final concentrations of 2 µM pentoxyresorufin (PR) and 0.1 mg microsomal protein/ml.

Plasma protein separation and $^{[125}I]$-T_4 competition binding

To determine the binding of the PCB-metabolite to plasma proteins *in vivo*, plasma samples from [^{14}C]-4-OH-CB107 treated animals (dams and fetuses) were separated by polyacrylamide gelelectrophoresis (PAGE) as described by Brouwer and Van den Berg (1986). In addition, the determination of [^{125}I]-T_4-competitive binding to specific plasma proteins was performed as described by Lans *et al.* (1993) and Darnerud *et al.* (1996). In short, plasma samples for gel slices (40 µl) were mixed 1:1 with a 50 mM Tris/38 mM glycine buffer (pH 8.3) containing 4.5% saccharose. Plasma samples for [^{125}I]-T_4 competition binding (25 µl) were incubated overnight with 100,000 counts per min [^{125}I]-T_4 (in 5 µl 50 mM Tris-HCl buffer, pH 8.0) at 4°C. Aliquots of 20 µl of the different samples were run on a 10% native separating gel for 4 hours at 4°C at a constant current of 50 mA. Each gel also contained plasma samples for protein staining (5 µl) and pure BSA and human TTR as a reference. After electrophoresis, the part of the gel containing the reference proteins was stained in 0.04% Coomassie Brilliant Blue in 3.5% perchloric acid for 60 min, and subsequently destained with 7% acetic acid for 24 h to determine the position of the proteins on the gel. The part of the gel for radioactivity measurements was frozen on the glass plate at –20°C overnight. The acrylamide gel was subsequently sliced into 1 mm pieces by a standardised procedure. Proteins in slices containing [^{14}C]-4-OH-CB107-derived radioactivity were first eluted by incubating the gel slices in tubes with 1 ml water overnight at 4°C. Four ml of scintillation fluid was added (Ultima Gold, Packard) the next day and the amount of radioactivity in each gel slice was quantified by LSC. Gel slices containing plasma samples incubated with [^{125}I]-T_4 were placed in RIA tubes and counted directly in a γ-counter (Cobra Auto Gamma Counter, Canberra Packard). The PAGE gel profile was made by plotting the [^{125}I]-T_4-radioactivity against the migration distance on the gel.

In vitro T_4-TTR competition binding study with 4-OH-CB107

The *in vitro* potency of 4-OH-CB107 to compete with T_4 for binding to human transthyretin was performed as described by Lans *et al.* (1993) with modifications (Meerts *et al.*, 2000; *Chapter 2*). Briefly, 30 nM human TTR, a mixture of [^{125}I]-labelled and unlabelled T_4 (70,000 cpm, 55 nM), and 4-OH-CB107 (in concentrations ranging from 10^{-9} to 10^{-4} M) were dissolved in 0.1 M Tris-HCl-buffer (pH 8.0, containing 0.1 M NaCl and 0.1 mM EDTA). The incubation mixture was allowed to reach binding equilibrium overnight at 4°C. Protein-bound and free [^{125}I]-T_4 were separated on 1 ml Biogel P-6DG columns and spin-forced eluted with 0.2 ml Tris-HCl buffer (1 minute at 100xg in a precooled centrifuge, Difuge, Hereaus). Radioactivity in the eluate containing the protein-bound [^{125}I]-T_4 was determined by gamma counting and compared to control incubations. The competition binding curves for T_4 and 4-OH-CB107 were made by plotting relative [^{125}I]-T_4-protein binding (% of control) against concentration competitor.

Statistical analysis

Data are presented as mean values (± SEM). Comparisons between two groups of animals were performed using Student's *t* test.

Results

Faecal and urinary [^{14}C]-4-OH-CB107 excretion

Faecal elimination of [^{14}C]-4-OH-CB107 derived radioactivity was high. After one day exposure (GD11), 15.1 ± 1.8% of the administered dose could be detected in the faeces (Figure 5.1), but at GD13 this level was raised to 60.6 ± 6.5% of the total dose administered.

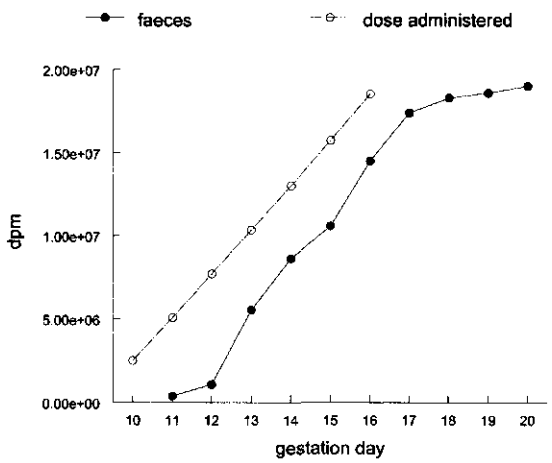

Figure 5.1. Cumulative faecal excretion of [^{14}C]-derived radioactivity from pregnant rats after oral exposure to 5 mg [^{14}C]-4-OH-CB107 per kg per day from gestation day 10 to 16. Data are expressed in dpm (result of one representative animal).

At GD17 and GD20, 78.4 ± 6.1% and 93.8 ± 6.9% of the total dose was excreted in the faeces, respectively. Urinary excretion was lower than 1% of the total given dose (data not shown). The average recovery of radioactivity per rat for animals dissected at GD17 or GD20 was 91.2 ± 6.3% and 97.2 ± 5.3%, respectively.

Tissue distribution of [^{14}C]-4-OH-CB107

In the pregnant rat, high levels of [^{14}C]-4-OH-CB107 derived radioactivity could be detected in plasma, liver and skeletal muscle on whole organ basis (Table 5.1). Organ levels in pregnant rats were higher at gestation day 17, i.e. one day after the last treatment, compared to gestation day 20. Significant decreases in radioactivity concentrations at GD20 could be detected in kidneys, thyroid and forebrain when levels were expressed in nmol per gram fresh weight, and in adrenals and forebrain when expressed in nmol per total organ.

Table 5.1. Distribution of [^{14}C]-4-OH-CB-107 derived radioactivity in maternal tissues at GD17 and GD20, after oral exposure to 5 mg/kg bw from GD 10 to 16.

Tissue/organ	nmol/g tissue or nmol/ml		nmol/organ	
Age	GD17	GD20	GD17	GD20
Plasma	39.02 ± 3.51	32.3 ± 5.09	415.5 ± 7.27	374.7 ± 6.90
Liver	4.85 ± 0.48	3.17 ± 0.07	49.08 ± 4.31	32.84 ± 1.44
Kidney	4.51 ± 0.4	2.86 ± 0.02*	7.70 ± 0.12	4.10 ± 0.17
Lung	4.27 ± 1.20	3.07 ± 0.62	4.96 ± 1.32	3.32 ± 0.87
Thyroid	3.79 ± 0.19	1.54 ± 0.18**	0.074 ± 0.02	0.04 ± 0.01
Thymus	3.22 ± 0.66	3.16 ± 0.89	1.05 ± 0.15	0.92 ± 0.29
Adrenals	3.12 ± 0.34	2.52 ± 0.06	0.23 ± 0.02	0.15 ± 0.003*
Pancreas	2.95 ± 0.35	2.16 ± 0.10	1.49 ± 0.20	0.73 ± 0.09
Forebrain	1.47 ± 0.08	0.96 ± 0.12*	1.84 ± 0.07	1.20 ± 0.14*
Cerebellum	1.52 ± 0.09	1.16 ± 0.34	0.68 ± 0.01	0.55 ± 0.18
Skeletal muscle	1.17 ± 0.25	0.28 ± 0.09	97.4 ± 12.1	30.8 ± 8.5
Abdominal fat	1.96 ± 0.04	1.18 ± 0.63	—	—
Brown adipose tissue	2.96 ± 0.19	2.19 ± 0.58	—	—

Data are expressed as nmol per g tissue (first columns) or nmol per total organ (last columns), and presented as mean ± standard error, n = 3 per time point. * Significantly different from GD17, $p < 0.05$; ** $p < 0.01$.

The distribution in the fetal compartment was different from that in dams (Table 5.2). There is a substantial accumulation of [^{14}C]-4-OH-CB107 derived radioactivity in the fetal compartment. The total radioactivity concentrations in the fetal compartment were 51.7 ± 3.2% of the total maternal concentrations. Significantly higher amounts of radioactivity could be detected in fetal liver, forebrain and cerebellum, whereas fetal plasma levels were comparable to maternal levels (Table 5.2).

Fetal/maternal liver ratios were as high as 15.9 ± 0.6 at GD17 and 11.0 ± 1.2 at GD20 (Table 5.3). In addition, although levels in maternal organs tend to decrease from gestation days 17 to 20, amounts of [^{14}C]-4-OH-CB107 in fetal liver and cerebellum increased when corrected for total tissue weight, though not significantly.

Table 5.2. Distribution of [^{14}C]-4-OH-CB-107 derived radioactivity in fetal tissues at GD17 and 20, after exposure of 5 mg/kg bw to the dams from GD 10 to 16.

Tissue/organ	**nmol/g tissue or nmol/ml**		**nmol/organs (pooled per mother)**	
Age	GD17	GD20	GD17	GD20
Plasma	n.a.	37.2 ± 5.14	—	66.6 ± 13.8$^{\#}$
Liver	89.41 ± 8.17$^{\#}$	35.12 ± 8.91$^{\#}$	58.7 ± 9.18	89.08 ± 10.1
Kidney	n.a.	5.28 ± 0.88	—	1.76 ± 0.05$^{\#}$
Lung	n.a.	4.08 ± 0.24	—	4.69 ± 0.56
Forebrain	3.11 ± 0.03$^{\#\#\#\#}$	1.54 ± 0.49	2.02 ± 0.11	1.69 ± 0.40
Cerebellum	2.87 ± 0.03$^{\#\#\#}$	2.63 ± 0.34	0.99 ± 0.02$^{\#\#\#\#}$	1.42 ± 0.24$^{\#}$
Placenta	6.22 ± 0.46	5.02 ± 1.01	35.8 ± 3.66	28.6 ± 5.41

Data are expressed as nmol per g tissue (first columns) or nmol per total organ (last two columns), and presented as mean ± standard error, n = 3 per time point.
n.a.: not analysed; $^{\#}$ significantly different from maternal levels at the same time point, $p < 0.05$; $^{\#\#\#}$ $p < 0.005$; $^{\#\#\#\#}$ $p < 0.0005$.

Body and organ weights

No effects were observed on maternal body weight gain, mean and total fetal body weight, number of implantation sites, resorptions, number of fetuses, or sex ratio (data not shown). In addition, absolute and relative organ weights from dams (liver, brain, kidneys, adrenals, thyroid, thymus, spleen, pancreas) and fetuses (liver, brain, kidneys, lungs) were not affected by maternal exposure to 5 mg 4-OH-CB107 per kg body weight from gestation days 10 to 16.

Plasma thyroid hormone levels

Thyroid hormone analysis revealed a significant decrease in maternal total thyroxine (TT_4) levels of 49% on GD17 and 38% on GD20 (Figure 5.2A) following exposure to 5 mg 4-OH-CB107 per kg body weight from GD10 to GD16. Maternal free thyroxine (FT_4, Figure 5.2B) and total triiodothyronine (TT_3, Figure 5.2C) levels were not significantly reduced. At GD20, fetal total thyroxine (TT_4) levels were drastically decreased by 89% and FT_4-levels were also significantly reduced by 41% after *in utero* exposure to the PCB-metabolite (Figure 5.3A,B).

Table 5.3. Fetal/maternal ratios of [^{14}C]-4-OH-CB-107 derived radioactivity at day 17 and 20 of gestation, after maternal exposure to 5 mg/kg bw from gestation day 10 to 16.

Tissue/organ	Foetal/maternal ratios[a)]	
Age	*GD17*	*GD20*
Plasma	—	1.2 ± 0.1
Liver	15.9 ± 0.6	11.0 ± 1.2
Kidney	—	1.8 ± 0.1
Lung	—	0.9 ± 0.1
Forebrain	2.1 ± 0.1	1.1 ± 0.1
Cerebellum	1.9 ± 0.1	2.6 ± 0.2

[a)] Fetal/maternal ratios were calculated with levels expressed as nmol/g or nmol/ml (see Table 5.2). Fetal samples were pooled per litter. N = 3 per time point and exposure group.

Fetal TT_4 levels on GD17 could only be detected in the control group (0.3 ± 0.1 nM). The level in 4-OH-CB107 exposed fetuses was below the detection limit of 0.09 nM, suggesting a decrease of at least 70% compared to the control group. Due to the small sample size these measurements could not be repeated. Fetal plasma levels of thyroid stimulating hormone (TSH) were significantly increased by 124% after 4-OH-CB107 treatment (Figure 5.4). Maternal TSH-levels were unchanged.

Brain thyroid hormone levels

At GD17, fetal cerebellum T_4 and T_3 levels were not significantly changed (Table 5.4). Forebrain T_4 levels at GD20 were significantly reduced by 35% in 4-OH-CB107 treated animals. Cerebellum T_4 levels at GD20 were also reduced, though not significantly (p = 0.051). No reductions in fetal T_3 levels could be detected in forebrain or cerebellum at GD20.

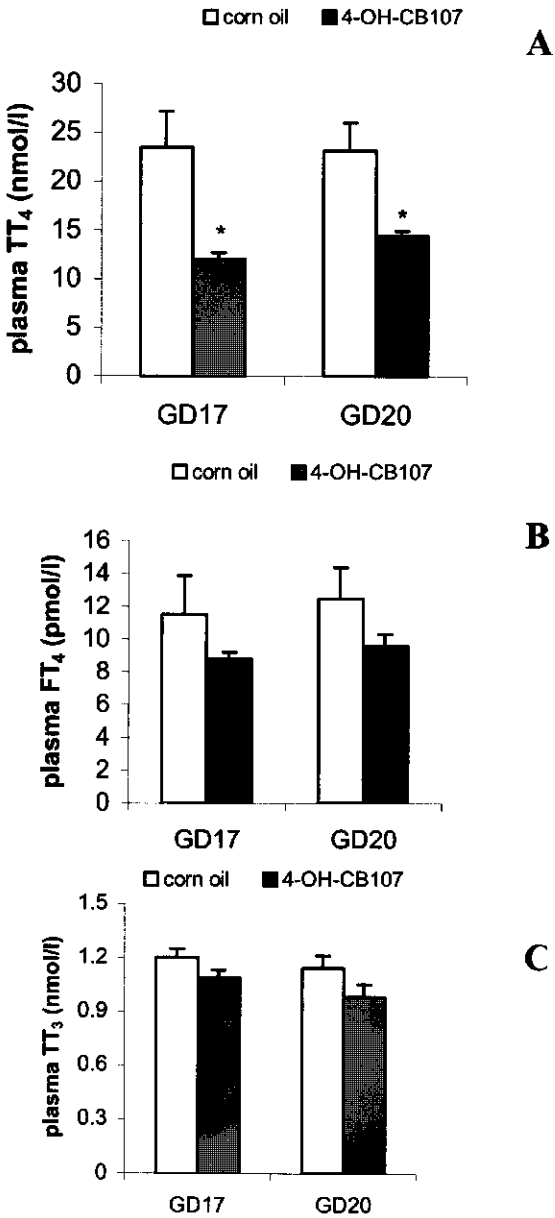

Figure 5.2. Plasma levels of maternal total thyroxine (TT_4, A), free thyroxine (FT_4, B) and total triiodothyronine (TT_3, C) after oral exposure to 5 mg 4-OH-CB107/kg bw from gestation days 10 to 16. Results are presented as mean ± SEM (n = 7). Statistically significant differences from controls in Student's t-test are given by * ($p < 0.05$).

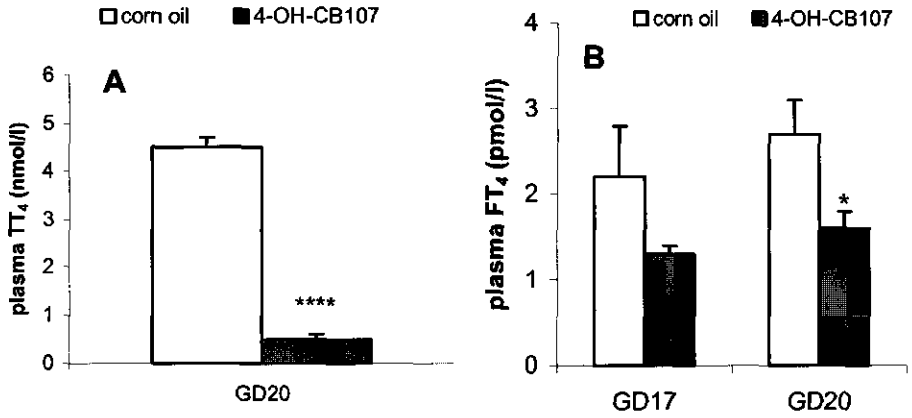

Figure 5.3. Plasma levels of fetal total thyroxine (TT_4, A) and free thyroxine (FT_4, B) after oral exposure to 5 mg 4-OH-CB107/kg bw from gestation days 10 to 16. Results are presented as mean ± SEM (n = 7). Statistically significant differences from controls in Student's t-test are given by * ($p < 0.05$) and **** ($p < 0.001$).

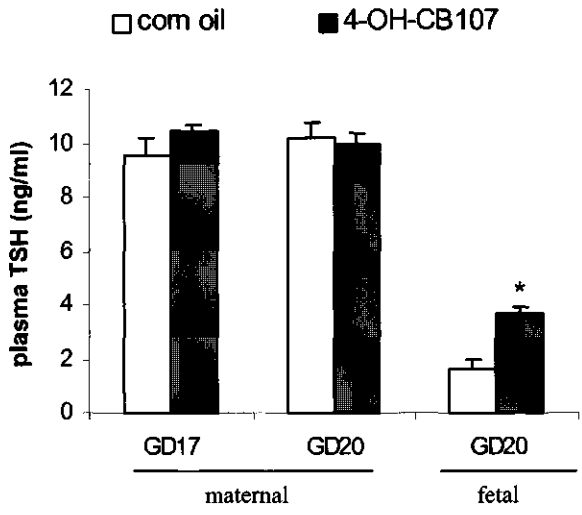

Figure 5.4. Plasma levels of thyroid stimulating hormone (TSH) in dams and fetuses following prenatal exposure of rats to 5 mg 4-OH-CB107 per kg bw from gestation days 10 to 16. Results are presented as mean ± SEM (n = 7 per exposure and time point). Statistically significant differences from controls in Student's t-test are given by * ($p < 0.05$).

Table 5.4. Fetal brain thyroid hormone levels.

Exposure	Corn oil	4-OH-CB107
	ng T_4 or T_3 per g tissue	
Gestation day 17		
Forebrain T_4	n.a.	n.a.
Forebrain T_3	n.a.	n.a.
Cerebellum T_4	0.53 ± 0.08 (6)	0.49 ± 0.06 (6)
Cerebellum T_3	0.14 ± 0.02 (6)	0.12 ± 0.01 (6)
Gestation day 20		
Forebrain T_4	1.79 ± 0.09 (4)	1.16 ± 0.07 (5) ****
Forebrain T_3	0.91 ± 0.06 (4)	0.80 ± 0.05 (6)
Cerebellum T_4	1.38 ± 0.13 (4)	1.10 ± 0.04 (5)
Cerebellum T_3	0.18 ± 0.02 (4)	0.16 ± 0.01 (6)

Data are presented as mean ± S.E.M. The number of animals is given in parentheses. n.a.: not analysed. **** Significantly different from corn oil, $p < 0.001$.

Thyroid hormone metabolism

Maternal and fetal hepatic microsomal type I deiodinase activities and T_4 uridine diphosphoglucuronosyl transferase activity (UDP-GT) were not altered by exposure to 4-OH-CB107 (data not shown).

The activity of brain type II 5'-thyroxine deiodinase (D-II) in forebrain homogenates from 17-day-old fetuses is very low compared to 20-day-old fetuses and maternal levels at GD17 and GD20. A significant increase of 67% compared to controls was observed at GD17 after exposure to 4-OH-CB107 (Figure 5.5). However, in 20-day-old fetuses, brain D-II levels were unaffected. D-II levels in maternal forebrain homogenates were decreased following exposure to 4-OH-CB107, though not significantly.

Ethoxy- and pentoxyresorufin-O-deethylase activity

No effects were detected on maternal and fetal hepatic microsomal ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD) activity (data not shown).

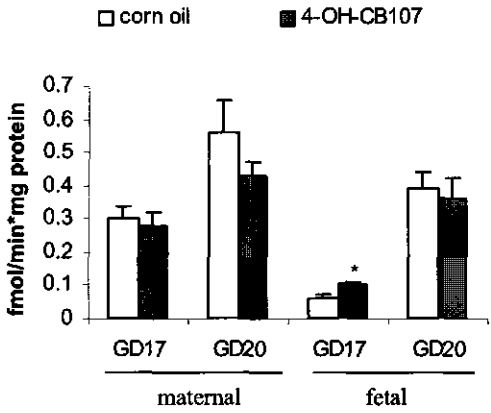

Figure 5.5. Type II thyroxine 5' deiodinase (D-II) activity in forebrain homogenates from dams and fetuses at gestation day 17 and 20, following prenatal exposure to 0 (corn oil) or 5 mg 4-OH-CB107 per kg bw from gestation days 10 to 16. Results are presented as mean ± SEM (n = 7). Statistically significant differences in Student's t-test are given by * ($p < 0.05$).

Plasma protein separation and [^{125}I]-T_4 competition binding

Plasma protein separation from animals treated with [^{14}C]-labelled 4-OH-CB107 revealed the binding of [^{14}C]-label to transthyretin in both maternal and fetal plasma (Figure 5.6). The identification of the transthyretin peak was based on co-migration of the TTR reference. *In vitro* T_4-competition binding with maternal and fetal plasma and separation of the plasma proteins by gel electrophoresis showed two peaks with [^{125}I]-T_4 bound radioactivity in maternal and three peaks with radioactivity in fetal plasma (Figure 5.7). The first peak is unbound radioactivity, which is left at the front of the gel. The second peak represented transthyretin, and the last one represented free T_4. The third peak, which was observed in fetal plasma only, could not be identified. The binding of [^{125}I]-T_4 with maternal plasma showed a significant decrease of 45% in the amount of [^{125}I]-T_4 bound to TTR in 4-OH-CB107 treated dams compared to controls (Figure 5.7a). The unbound radioactivity can be detected at the front of the gel. In fetal plasma, this shift in the position of radioactivity is not very clear (Figure 5.7b). The amount of [^{125}I]-T_4 bound to TTR in fetuses treated *in utero* with 4-OH-CB107 was slightly though not significantly decreased.

In vitro T_4-TTR competition binding study with 4-OH-CB107

The binding affinity (K_a) and IC_{50}-value of 4-OH-CB107 as determined in the *in vitro* T_4-TTR competition binding assay were 1.19 (± 0.01) x 10^8 M^{-1} and 24.4 ± 2.2 nM, respectively (Figure 5.8). The relative potency compared to the natural ligand T_4 (IC_{50} of 80.7 nM) was 3.3 ± 0.3.

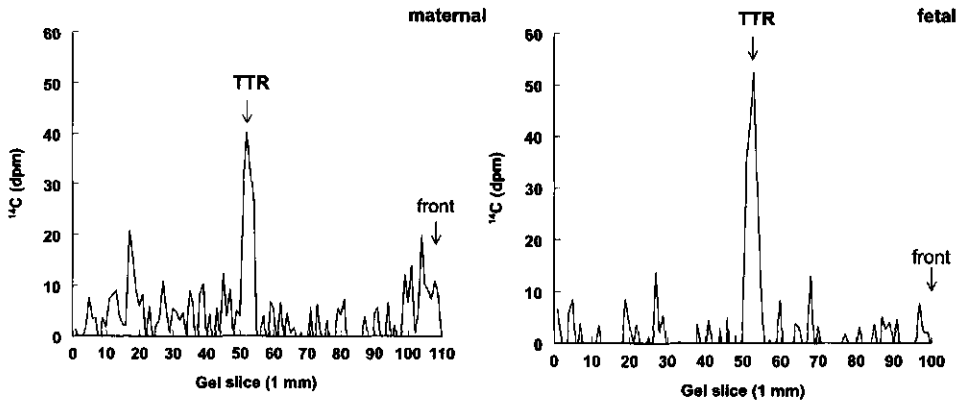

Figure 5.6. Distribution of [^{14}C]-derived radioactivity in maternal (left) and pooled fetal (right) plasma at gestation day 20 after native polyacrylamide gelelectrophoresis. Pregnant rats were exposed to 5 mg [^{14}C]-labelled 4-OH-CB107 per kg bw from gestation days 10 to 16.

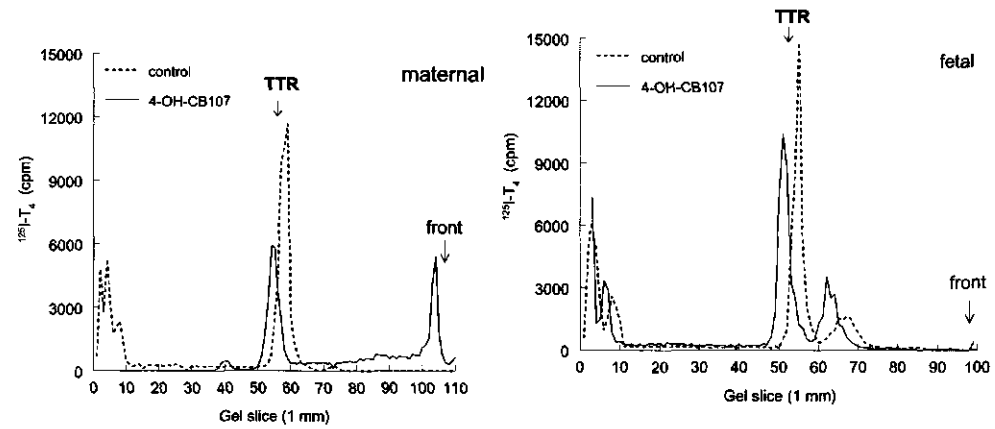

Figure 5.7. Distribution of [^{125}I]-T_4-derived radioactivity in maternal (left) and pooled fetal (right) plasma after in vitro incubation with [^{125}I]-T_4 and native PAGE. Pregnant rats were treated with corn oil (dotted lines) or 5 mg 4-OH-CB107 per kg body weight from gestation days 10 to 16.

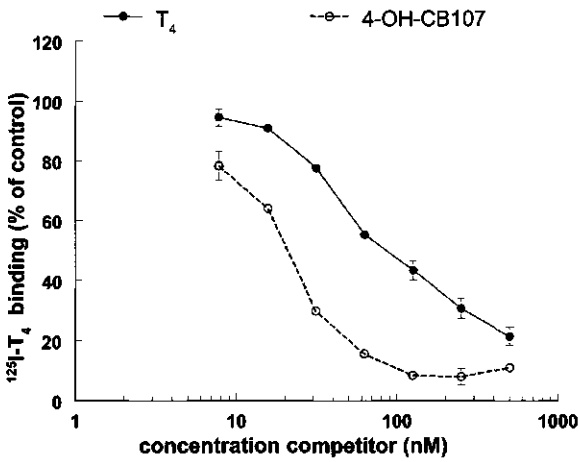

Figure 5.8. Displacement of T_4 from TTR by 4-OH-CB107. Data points are mean values ± standard deviation of one representative measurement in duplicate. If no error bar is visible, it is smaller than the marker. Relative [^{125}I]-T_4-TTR binding is presented as % of control value.

Discussion

The results of the present study show that maternal exposure to the PCB-metabolite 4-OH-2,3,3',4',5-pentaCB from gestation days 10 to 16 results in considerable transfer of this metabolite from the mother to the fetus, thereby affecting both maternal and especially fetal thyroid hormone levels. Detection of 4-OH-CB107 bound to transthyretin in fetal and maternal plasma suggests that binding of a compound to TTR *in vivo* can lead to facilitated maternal to fetal transfer, decreased maternal and fetal plasma T_4 levels and decreased fetal brain T_4 levels. Hepatic UDP-glucuronosyltransferase (UDP-GT) levels were not induced in dams or fetuses, indicating that this mechanism did not play a role in the observed decrease in plasma thyroid hormone levels as shown for e.g. TCDD and parent PCB-compounds (Van Birgelen *et al.*, 1995; Darnerud *et al.*, 1986).

The internal dose of [^{14}C]-4-OH-CB107 in pregnant dams was low, since most of the radioactivity was excreted in the faeces. However, relatively high levels of [^{14}C]-4-OH-CB107 derived radioactivity could be detected in the fetal compartment (i.e. 52% of the total maternal concentrations), indicating a high placental transfer of the compound. At gestation days 17 and 20, fetal liver, forebrain and cerebellum levels were all higher than maternal levels, whereas fetal plasma levels were almost equal to maternal plasma levels (fetal/maternal ratio of 1.16 ± 0.03 at GD20). The approximate 16- and 11-fold higher levels in fetal livers at gestation day 17 and 20, respectively, compared to maternal livers are striking. This may be due to the fact that the liver is one of the major sites of TTR synthesis in the body (Dickson *et al.*, 1985). In addition, the 3.3 fold higher affinity of 4-OH-CB107 for TTR *in vitro* compared to the natural ligand T_4 (this study) and the observed *in vivo* binding of [^{14}C]-4-OH-CB107 (this study) derived radioactivity are in line with this explanation.

Significant reductions (approximately 90%) in fetal plasma TT_4 levels at GD20 could be

detected after maternal exposure to 4-OH-CB107, with fetal plasma metabolite levels of 12.7 µg/g on wet weight basis. As a comparison, rats exposed to 5 or 25 mg/kg Aroclor 1254 in the same experimental setup resulted in a 52% or 74% decrease in fetal plasma TT_4, respectively, with fetal plasma 4-OH-CB107 concentrations of approximately 0.6 and 1.6 µg/g (Morse *et al.*, 1996a). The higher decreases in fetal free T_4 levels and maternal total and free T_4 levels after Aroclor 1254 exposure can be explained by the additional induction of hepatic UDP-GT by Aroclor 1254 and consequently induced biliary excretion of T_4 after glucuronidation. Surprisingly, exposure of pregnant mice to one single i.v. dose of 50 µmol (= 17.1 mg) per kg body weight of another PCB-metabolite, 4-OH-2',3,3',4',5-pentaCB (5.9 fold higher affinity for TTR *in vitro* compared to T_4, Lans *et al.*, 1993) resulted in an only 14% reduction of fetal plasma T_4 levels compared to control levels (Sinjari and Darnerud, 1998). However, comparison with this latter study is difficult, since the route of exposure, species and time point of analysis were all different.

Fetal plasma thyroid stimulating hormone levels were significantly increased at GD20, indicating that the hypothalamus-pituitary-thyroid (HPT) axis was stimulated in the fetuses. This was expected, since reductions in plasma T_4 levels are responsible for regulating fetal plasma TSH levels (Morreale de Escobar *et al.*, 1993). However, stimulation of this HPT axis occurs at a stage when the setpoint of homeostatic control is being developed, and it is possible that these disturbances might have a prolonged effect on the homeostatic control of thyroid hormones in these animals. Morse *et al.* (1996a) reported normal levels of plasma thyroid hormones in offspring exposed prenatally to 5 mg/kg Aroclor 1254 at day 21 postpartum, but a statistically significant elevation of plasma TT_4 levels in male offspring at 90 days postpartum.

Despite the very low T_4 levels in fetal plasma, fetal brain T_4 levels were reduced only in forebrain and cerebellum homogenates at GD20, and not at GD17. It should be stated however, that brain T_4 and T_3 levels at GD17 were very difficult to measure because of small sample sizes, and we only used cerebellum samples at GD17 for thyroid hormone analysis. No changes were observed in brain T_3 levels at GD17 or GD20. The induction of brain type II 5'-thyroxine deiodinase (D-II) is a well known response of the rat brain to maintain brain T_3 levels when circulating T_4 concentrations are decreased (Silva and Matthews, 1984; Ruiz de Oña *et al.*, 1988; Obregón *et al.*, 1984), and has been reported before in fetal and neonatal rats after maternal exposure to 3,3',4,4',5,5'-hexachlorobiphenyl (Morse *et al.*, 1993) and Aroclor 1254 (Morse *et al.*, 1996a). Maternal exposure to Aroclor 1254 caused a significant decrease in fetal forebrain T_3 levels only after exposure to 5 mg/kg bw, and not to 25 mg/kg body weight per day.

The accumulation of 4-OH-CB107 in fetal forebrain and cerebellum may have an effect on the neurodevelopment of the offspring. In a comparable study by Morse *et al.* (1996a), exposure of pregnant rats to Aroclor 1254 from GD10 to 16 resulted in long term alterations in glial and neuronal cell marker proteins in the offspring (Morse *et al.*, 1996b), and significant increases in 5-hydroxytryptamine (5-HT) metabolism (Morse *et al.*, 1996c). These adverse effects were likely caused by 4-OH-CB107, since this metabolite accumulated in fetal brains after maternal exposure to Aroclor 1254. Concentrations of 4-OH-CB107 in fetal brains (determined by GC/MS analysis) at GD20 were approximately 0.16 ppm on fresh weight basis (Morse *et al.*, 1996a). In the current study, maternal exposure of rats to 5 mg/kg 4-OH-CB107 resulted in concentrations of 0.90 ppm 4-OH-

CB107 in fetal cerebellum and 0.53 ppm in fetal forebrain. In total, the brain 4-OH-CB107 levels in this study were approximately 9 times higher compared to maternal exposure to 25 mg/kg Aroclor 1254. Therefore, we performed another study to investigate the effects of maternal 4-OH-CB107 exposure on the development and behaviour of the offspring in more detail. These results will be presented in a separate publication (see *Chapter 7*).

OH-PCBs can also exert several other effects on the endocrine system. OH-PCBs have been reported to interact with thyroid hormone metabolising enzymes, such as iodothyronine 5'-deiodinase (Adams *et al.*, 1990; Lans, 1995; Rickenbacher *et al.*, 1989) and iodothyronine sulfotransferase (Schuur *et al.*, 1998) *in vitro*. In addition, some OH-PCBs competitively bind to the estrogen receptor (ER) and exhibit estrogenic activity in the mouse uterus (Korach *et al.*, 1988). Recently, Kester *et al.* (2000) demonstrated that various environmentally relevant OH-PCBs were extremely potent inhibitors of human estrogen sulfotransferases.

In conclusion, exposure of pregnant rats to the PCB-metabolite 4-OH-CB107 results into drastic reductions in fetal plasma thyroid hormone concentrations, and to an accumulation of the compound in fetal liver, brain and plasma. It is suggested that the observed binding of 4-OH-CB107 to TTR may play a role in the retention of the metabolite in plasma, in the maternal to fetal transport and in the distribution of 4-OH-CB107 in the fetal compartment. The question remaining is whether this possible mechanism is also operating in humans. Even though in humans thyroxine binding globulin is the main thyroid hormone transport protein in the blood, TTR still plays a role in mediating the delivery of T_4 across the blood-brain barrier, transporting T_4 into the cerebrospinal fluid and transferring maternal-to-fetal T_4 over the placenta (Calvo *et al*, 1990; Southwell *et al*, 1993). In fact, current determinations of 4-OH-CB107 levels in human maternal plasma and cord blood show approximately three fold higher levels in cord blood (Bergman *et al.*, 1999b), suggesting that indeed transport of OH-PCBs to the human fetus is possible. If this facilitated transport is also operating for other organohalogen compounds, further investigation is needed into the possible consequences of exposure to these compounds on neuronal development of the offspring.

Acknowledgements

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CHAPTER 6

Effects of *in utero* exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) on developmental landmarks, steroid hormone levels and female estrous cyclicity in rats

Abstract

Previous studies at our laboratory revealed that the polychlorinated biphenyl metabolite 4-OH-2,3,3',4',5-pentaCB (4-OH-CB107), one of the major metabolites of PCBs detected in human blood, accumulated in fetal liver, brain and plasma and reduced maternal and fetal thyroid hormone levels after prenatal exposure to pregnant rats from gestation days 10 to 16 (*Chapter 5*). Recently, we started to investigate the effects of 4-OH-CB107 on behavioral and reproductive development of rat offspring from postnatal days 0 to 310. In this paper, the effects on estrous cycle and steroid hormone levels are reported. Effects caused by 4-OH-CB107 only were compared with effects observed by the parent compound mixture Aroclor 1254, which was found to give rise to an accumulation of mainly 4-OH-CB107 in fetuses following maternal exposure.

The most pronounced 4-OH-CB107 related developmental effects were seen on female estrous cyclicity and plasma estradiol concentrations. A significant and dose dependent prolongation of the estrous cycle length (4 to 7 days) was observed in 75% (0.5 mg/kg 4-OH-CB107) and 82% (5.0 mg/kg 4-OH-CB107) of female offspring determined between the age of 210 to 231 days, compared to 64% of Aroclor 1254 (25 mg/kg) exposed offspring. Prolongation was primarily due to a prolongation of the diestrous stage, resembling a state of pseudopregnancy. Nevertheless, reproductive capabilities of female F_1 offspring appeared to be normal. Strikingly, plasma estradiol concentrations in female rat offspring at the age of 11 months were significantly increased (by 50%) in the proestrous stage after exposure to 5 mg 4-OH-CB107 per kg body weight. No effects on estradiol levels were observed in Aroclor 1254 treated animals.

In males, no effects were observed on preputial separation during development, male accessory sex organ weights (prostate, testis, seminal vesicle and cauda epididymis) or testosterone levels at the age of 11 months. These results indicate that *in utero* exposure to 4-OH-CB107 leads to reproductive changes that may reflect early signs of reproductive senescence in female offspring at a relative early stage in life. The possible impact on neurobehaviour following exposure to 4-OH-CB107 will be reported elsewhere.

Based on: Meerts I.A.T.M., Hoving S., van den Berg J.H.J., Weijers B.M., Swarts H.J., van der Beek E.M., Bergman Å, Koeman J.H., and Brouwer A. *Submitted.*

Introduction

Polychlorinated biphenyls (PCBs) are environmental contaminants causing a broad range of toxic effects (reviewed in Brucker-Davis, 1998; Brouwer *et al.*, 1998; Peterson *et al.*, 1993; Safe, 1990, 1994; Seegal, 1996; Tilson and Kodavanti, 1997). Dependent on the number and position of the chlorine substituents, PCBs can be metabolized to hydroxylated PCBs (OH-PCBs) in animals via an arene oxide intermediate, catalyzed by cytochrome P450s 1A and 2B (Safe, 1994). Hydroxylated metabolites of PCBs have been identified in the blood of marine mammals, polar bears, fish-eating birds and humans (Bergman *et al.*, 1994; Klasson-Wehler *et al.*, 1998; Sandau *et al.*, 2000; Sjödin *et al,*, 2000), at concentrations of 10-30% of the PCB concentration in human blood (Sandau et al 2000, Sjödin et al 2000) but as high as 2-3 times the PCB level in Polar bear blood (Sandau, 2000). Several potentially adverse effects of OH-PCBs on the endocrine system have been reported. Hydroxylated PCBs present in human plasma were shown to competitively inhibit binding of the natural thyroid hormone thyroxine (T_4) to its transport protein transthyretin (TTR) (Lans *et al.*, 1993, 1994). In addition, the activities of hepatic type I iodothyronine deiodinase (ID-1) and iodothyronine sulfotransferases (both enzymes involved in the intracellular metabolism of thyroid hormones) were inhibited by OH-PCBs (Adams *et al.*, 1990; Lans *et al.*, 1993; Rickenbacher *et al.*, 1989 and Schuur *et al.*, 1998).

The binding of OH-PCBs to TTR *in vivo* is thought to facilitate the transport of this metabolite across the placenta from the mother to the fetus, thereby affecting maternal but especially fetal thyroid hormone levels (reviewed by Brouwer *et al.*, 1998). Earlier studies at our laboratory showed a selective accumulation of the PCB-metabolite 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) in fetal and neonatal rats after maternal exposure to Aroclor 1254 from gestation day 10 to 16 (Morse *et al.*, 1996a). Thyroid hormone levels in the exposed fetuses and neonates were significantly decreased. It was hypothesized that the decrease in plasma thyroid hormone levels was caused by the competitive binding of 4-OH-CB-107 to TTR, which was observed for this compound *in vitro* (Lans, unpublished results, Meerts *et al.*, submitted, *Chapter 5*). In a recent study we were able to support this hypothesis (Meerts *et al.*, submitted, *Chapter 5*). Exposure of pregnant rats to ^{14}C-radiolabelled 4-OH-CB107 from gestation days 10 to 16 resulted in a selective accumulation of 4-OH-CB107 in fetal livers, brain and plasma, measured at gestation days 17 and 20. Polyacrylamide gel electrophoresis of maternal and fetal plasma revealed the binding of 4-OH-CB107 to TTR. Consequently, maternal but especially fetal total thyroxine (TT_4) levels at gestation day 20 were significantly decreased by 38% and 89%, respectively.

A prenatal or early postnatal hypothyroid status is known to severely affect the normal development of the brain and sexual organs. Effects on brain development include disorders of neuronal process growth (Stein *et al.*, 1991), disruption of the expression pattern of neurotrophins, nerve growth factor, and brain derived neurotrophic factor (Nevue and Arenas, 1996), and interference in neurotransmitter systems (Seegal, 1996). Several of these effects have also been observed following *in utero* and lactational exposure of rats to PCBs. Exposure of rats to Aroclor 1254 resulted in alterations in regional brain serotonin metabolism and in glial and neuronal cell markers (Morse *et al.*, 1996c). Exposure of rats to Aroclor 1016 from gestation day 8 through

weaning caused elevations in regional dopamine concentrations in rat offspring (Seegal, 1992, 1994). Studies with individual PCB congeners revealed that the structure of the congener and the age of the animal at the time of exposure were important variables for the observed effects on brain dopamine levels.

In addition to the above mentioned effects of PCB-induced hypothyroidism on brain development, it is also possible that the relatively high concentrations of hydroxylated PCB congeners in plasma or brain of fetal rats have a direct effect on brain development and/or reproduction. Hydroxylated PCBs are known to induce uncoupling of oxidative phosphorylation in mitochondria (Lans *et al.*, 1990; Narasimhan *et al.*, 1991), and inhibition of intercellular communication (de Haan *et al.*, 1994). Some hydroxylated PCBs also possess (anti-) estrogenic activities (Korach *et al.*, 1988, Moore *et al.*, 1997). The OH-PCBs identified in human serum were mostly anti-estrogenic (Moore *et al.*, 1997). Recently, Kester *et al.* (2000) reported extremely potent inhibition of human estrogen sulfotransferase activity *in vitro* by environmentally relevant OH-PCBs, suggesting that OH-PCBs indirectly induce estrogenic activity by increasing estradiol bioavailability in target tissues.

The aim of the current study was to investigate the potential impact of *in utero* exposure to 4-OH-CB107 on the development of rat offspring and the possible long-term effects on sex steroid hormone levels and reproduction. Effects on brain development were also investigated, but will be reported elsewhere (see *Chapter 7*). Pregnant rats were exposed to 0.5 or 5 mg of 4-OH-CB107 from gestation days 10 to 16. To discriminate between the effects caused by parent compounds and hydroxy-metabolites one group of animals was dosed with the parent compound mixture Aroclor 1254.

Animals, Materials and Methods

Chemicals

4-Hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) was synthesized as described by Bergman *et al.* (1995) and at least 99.9% pure. The nomenclature used is adapted from Letcher *et al.* (2000). Aroclor 1254 was kindly donated by Prof. Dr. M. van den Berg (Ritox, University of Utrecht, The Netherlands). Dichloromethane and Tris were purchased from Merck Chemical Company (Darmstadt, Germany). ^{125}I-Estradiol, estradiol antiserum and goat anti rabbit gamma globulin were obtained from Diagnostic Products Corporation (DPC, Breda, The Netherlands). Pregnen-(4)-dion-(3,20), 17β-estradiol and bovine serum albumin were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). [1,2,6,7-^{3}H]-progesterone was purchased from Amersham (Buckinghamshire, UK). Progesterone antiserum was produced as described in Van der Meulen *et al.* (1988) and Mattheij and Swarts (1995). Testosterone was obtained from DRG (Marburg, Germany). Ultima Gold liquid scintillation fluid was purchased from Canberra Packard (Packard, St. Louis, MO, USA).

Animals and treatment

All experimental procedures involving animals were approved by the Animal Welfare Committee of Wageningen University. Wistar WU rats (60 females, 30 males; 14 weeks old) were purchased from Charles River (Sulzfeld, Germany) and allowed to acclimatize for three weeks. Throughout the

experiment, animals were kept in an artificial 12-h:12-h light-dark cycle with lights on at 06:00 h. Room temperature was maintained at 21 ± 2°C and humidity at 50 ± 10%. Animals were provided rat chow (Hope Farms, Woerden, the Netherlands) and tap water *ad libitum*.

After the acclimatization period two females were placed in a cage with one male from 17:00 to 8:00 hr the next day. Copulation was examined each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, this day was designated as day 0 of gestation (GD0) and females were housed individually. On day 10 of gestation the pregnant rats were divided randomly into the different treatment groups and transferred to a macrolon, stainless steel cage to facilitate the collection of PCB-contaminated feces. In total 52 pregnant rats (13 per exposure group) were dosed by oral intubation with 0, 0.5 or 5 mg 4-OH-CB107 per kg body weight per day dissolved in corn oil (2 ml/kg) from gestation days 10 to 16. For comparison of the effects of 4-OH-CB107 with effects caused by parent PCB congeners, a fourth group of rats was dosed with 25 mg Aroclor 1254 per kg body weight from gestation days 10 to 16. In a former study it was observed that this dose level of Aroclor 1254 resulted in an amount of 4-OH-CB107 in the fetal compartment in the same range as observed after *in utero* exposure to 5 mg/kg 4-OH-CB107 (Meerts *et al.*, *Chapter 5*).

Maternal body weights were monitored daily throughout gestation. On day 20 of gestation, pregnant females were transferred to bedding material. At birth, i.e. postnatal day (PND) 0, live offspring were counted and sexed. Individual pups and the dams were weighed on PND 1, 4, 7, 14, and 21, and after weaning body weights of the offspring were monitored weekly until sacrifice. On PND 4, litters were adjusted to 4 males and 4 females. Generally, this required the termination of excess offspring. However, in a few cases where a litter contained less than eight pups or the sex distribution was not permissive, the standardized litter required pups from two dams. To maintain litter independence, no dam was allowed to contribute pups to more than one litter. In addition, pups transferred from one litter to another were not used for any analysis. The standardized litter became the experimental unit and all treatment mean values reported are litter based. Liver, kidneys, brain and thymus were weighed. The remaining offspring were numerically marked on their feet to identify individual animals within a litter.

Developmental landmarks

During the study, a number of developmental landmarks of all litters were recorded in a blind fashion. Treatment groups were decoded only after termination of the animals when all analyses were completed. On PND 1 and PND 4, anogenital distance (AGD) and crown-rump length (CRL) were measured on each pup by means of a micrometer, capable of resolution to 0.01 mm. AGD was measured in both sexes as the distance from the anterior edge of the anus to the base of the genital tubercle. Measurements of AGD and CRL were done by one person, to avoid individual variations. Each individual pup was additionally examined for the following developmental landmarks: pinna detachment (starting on PND 1), age at the onset of hair growth, age at bilateral eye opening (starting on PND 12).

Following weaning at PND 21, pups were housed with littermates in unisexual groups, two pups per cage. Dams were sacrificed at PND 21 under ether anaesthesia and blood was collected via the vena cava in heparinized tubes for thyroid hormone measurements. Liver, kidneys, adrenals,

thymus, brain, spleen, uterus and ovary were collected, blotted dry and weighed. All organs were frozen in liquid nitrogen and stored at –80°C.

Female pups were examined daily for vaginal opening, starting at PND 30. The age at preputial separation (Korenbrot *et al.*, 1977) in male offspring was examined from PND 35 until a complete preputial separation in all males was achieved. After puberty, the offspring was split into two cohorts; one cohort (n = 41 litters with 2 males and 2 females per litter) was housed individually and used for behavioural studies which will be reported elsewhere. From the other cohort (n = 41 litters, 2 males and 2 females per litter), females were also housed individually to study estrous cyclicity and reproduction as described below. Male offspring from this cohort were housed in unisexual groups with 2 animals per cage until dissections at about 11 months of age.

Reproductive capability of female offspring

Female vaginal estrous cyclicity was monitored for 21 days, starting at PND210, by daily evaluation of the vaginal smears (between 8:00 and 10:00 hr and at other times as needed). Differentiation of the cells during the four days of the estrous cycle was determined microscopically according to Staples and Geils (1965). Due to the effects found on the length of the estrous cycle (cf. Results), a study was conducted to determine the reproductive capability of the female offspring. The females were split into two cohorts; one cohort stayed in unisexual groups with 2 females per group until necropsy at about 12 months of age to determine possible long term adverse effects on sex steroid parameters. The other cohort (n = 41) was housed individually, and after 2 weeks mating with untreated males (16 weeks old, Charles River, Sulzfeld, Germany) was started (1:1). Copulation was examined each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, this day was designated as day 0 of gestation (GD0). If no spermatozoa were found, the female was remated up to two weeks with a stud male. The number of matings was recorded. Pregnant females were sacrificed at GD20 under ether anaesthesia. Maternal blood was collected via the vena cava in heparinized tubes, and plasma was prepared and stored at –80°C for measuring thyroid hormones, estradiol, progesterone and testosterone. Maternal body weight, ovarian weights, and the number of corpora lutea (examined with a microscope), implantation sites and embryos were recorded. Additionally, maternal liver, kidneys, spleen, brains and thymus were weighed. From the fetuses, sex was determined and liver, brain and thymus were removed and weighed.

Long term effects on male and female hormone levels

Male offspring and the female cohort that was not used for the reproductive capability study were dissected at about 11 months of age (PND 310 - 320) to determine possible long term adverse effects on sex steroid parameters. To avoid the effects of stress on serum steroid hormone levels, the animals were killed by decapitation within 15 s of removal from their cages. Dissections were conducted between 08:00 and 12:00 to minimize circadian influences on testosterone and estradiol levels. Trunk blood was collected in eppendorf tubes (for serum preparation) and heparinized tubes (for plasma) for hormone analysis. Immediately after collection of the blood, the brains were dissected rapidly (within 5 minutes) and separated in different regions for neurotransmitter analyses (described elsewhere). Weights measured at dissection included body, liver, kidneys, adrenals, spleen, thymus and pituitary

gland. From the males, testes, prostate, seminal vesicle and cauda epididymis were weighed additionally. The coagulating glands were detached from the seminal vesicles and care was taken to avoid expression of fluids from these organs. From the females, uteri and ovary were weighed. Estrous cycle stage was estimated at dissection from the appearance of the uterus as either estrous ('ballooning') or non-estrous.

Measurement of sex steroids

Before estradiol measurements, 100 µl plasma was extracted three times with 1.25 ml dichloromethane in glass tubes by vortexing for 30 s, centrifugation at 1,000xg for 5 minutes and collection of the dichloromethane phase. The dichloromethane phases were pooled, evaporated to dryness in a Savant Speed Vac vacuum concentrator and 175 µl phosphate buffered saline containing 0.1% (w/v) BSA was added to each tube. After thoroughly vortexing, estradiol concentrations were measured in triplicate as described by Palm *et al.* (1999). The extraction efficiency, determined by the addition of tracer amounts of ^{125}I-E_2 before extraction of the plasma, was 94.2 ± 2.84% for plasma from pregnant rats and 95.5 ± 2.32% for non-pregnant rats.

Progesterone concentrations were measured in triplicate in unextracted plasma, diluted 20 times in phosphate buffered saline containing 0.1% (w/v) BSA, as described by Van der Meulen *et al.* (1988) and Mattheij *et al.* (1995). Testosterone concentrations were measured in duplicate in extracted serum using a commercial ELISA kit (DRG, Marburg, Germany). Testosterone was extracted from 200 µl serum using the extraction method described above for estradiol analysis. The extraction efficiency was determined by comparing extracted standard samples with the non-extracted standards and was 95.3 ± 0.6%.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package. Differences between the number of pups and organ weights were analyzed by means of analysis of variance (ANOVA). Levene's test was used to evaluate homogeneity of variances, and the Bonferroni test was used to compare individual treatment means when ANOVA indicated that significant differences were present. For the evaluation of body weight development until sacrifice, ANOVA with repeated measures was used, with age as factor. For hormone determinations, one pup per litter was used. Nonparametric analysis used the Kruskal-Wallis ANOVA by ranks. When this indicated significant differences, treatment ranks were compared to the control group by the Wilcoxon-Mann-Whitney test. Categorical data were analyzed by Chi-square analysis.

In all cases, the litter was the independent experimental unit and data from individual male and female offspring were assumed to be representative of the litter. Where more than one male or female from a given litter was evaluated, the results were averaged to form a litter mean. In all cases significance was set at $p < 0.05$.

Results

P0 Fertility

Of the 52 exposed females, 11 were false positive (i.e., spermatozoa were found on GD 0 but the female was not pregnant). Unfortunately, 5 false positive females were randomly assigned to the lowest 4-OH-CB107 exposure group (0.5 mg/kg), resulting in only 8 litters in this group. The corn oil, 4-OH-CB107 (5 mg/kg) and Aroclor treated groups all contained 11 litters (Table 6.1). All pregnant dams delivered without complications, and no effects could be observed on length of gestational period, litter size, sex ratio (Table 6.1), number of live fetuses, late gestational death, number of resorptions or postnatal death (data not shown). In addition, treatment of dams with 4-OH-CB107 or Aroclor 1254 caused no overt signs of toxicity in dams and offspring as assessed by visual inspection (data not shown).

Growth and Development

Male and female body weight gain of the offspring were slightly, but not significantly, reduced by maternal exposure to Aroclor 1254 (data not shown). At PND 4, male body weights from 4-OH-CB107 treated animals were significantly higher ($p < 0.01$) compared to Aroclor treated animals (Table 6.1). Crown-rump lengths (CRL) of male and female offspring exposed to 0.5 mg 4-OH-CB107 per kg body weight were significantly higher compared to Aroclor treated offspring. When corrected for crown rump lengths of the animals, female anogenital distances (AGD/CRL-ratio) of the Aroclor treated animals were significantly increased by 16% compared to controls (Table 6.1).

The onset of bilateral eye opening was significantly earlier in male and female offspring exposed to Aroclor 1254 via the dams. The day of vaginal opening was not changed by either 4-OH-CB107 or Aroclor 1254 exposure (Table 6.1). In all groups, vaginal opening occurred at approximately PND 34-36 (8-11 litters per exposure group). Male preputial separation was completed at PND 43-44 in all treatment groups.

Organ weights of dams (P_0) and neonates (F_1)

At PND 21, maternal body weights and absolute or relative weights of the collected organs (liver, kidneys, adrenals, thymus, brain, spleen, uterus, ovary) of treated animals showed no differences compared to the control group. Absolute and relative liver weights from male and female offspring at PND 4 were significantly increased following exposure to Aroclor 1254 (44% and 38%, respectively, for relative liver weights, Table 6.2). Relative thymus weight at PND 4 was significantly reduced in both male (23%) and female (27%) offspring exposed to Aroclor 1254 *in utero*.

Estrous cyclicity in F_1

The average estrous cycle length of female offspring (F_1) monitored at the age of 210 to 231 days was significantly prolonged in females exposed *in utero* to 5 mg 4-OH-CB107 per kg body weight (Figure 6.1A). The prolongation of the estrous cycle was caused by an increased length to 4-7 days of the diestrous stage (normal length 2 days), which lasted more than 4 days in 50% and 64% of the females in the 0.5 and 5 mg/kg 4-OH-CB107 treatment group, respectively (Figure 6.1B).

Table 6.1. Effects on developmental landmarks in rat offspring following in utero exposure to 4-OH-CB107 or Aroclor 1254 from gestation days 10 to 16.

Parameters		Control	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
No. of litters		11	8	11	11
Litter size		11.7 ± 0.3	10.9 ± 0.3	10.9 ± 0.4	10.5 ± 0.4
Gestational period		21.5 ± 0.1	21.8 ± 0.1	21.6 ± 0.2	21.7 ± 0.1
Male to female ratio (%)		1.26 ± 0.14	1.30 ± 0.53	1.27 ± 0.24	1.00 ± 0.17
Body weight PND 4	Male	8.60 ± 0.16	9.20 ± 0.26$^{\#\#}$	9.50 ± 0.46$^{\#\#}$	8.11 ± 0.17
	Female	8.29 ± 0.18	8.87 ± 0.25	9.08 ± 0.41	8.20 ± 0.17
AGD[a] PND 4	Male	3.69 ± 0.12	3.87 ± 0.17	4.04 ± 0.08$^{\#}$	3.63 ± 0.12
	Female	1.52 ± 0.08	1.64 ± 0.06	1.64 ± 0.05	1.74 ± 0.07
CRL[b] PND 4	Male	49.50 ± 0.42	50.52 ± 0.52$^{\#}$	50.52 ± 0.68	48.33 ± 0.28
	Female	48.74 ± 0.41	49.51 ± 0.53$^{\#}$	49.44 ± 0.58	47.11 ± 0.34
AGD/CRL ratio	Male	0.074 ± 0.002	0.074 ± 0.004	0.080 ± 0.001	0.077 ± 0.002
	Female	0.031 ± 0.002	0.033 ± 0.001	0.033 ± 0.0001	0.036 ± 0.001*
Pinna detachment[c]	Male	3.8 ± 0.2	4.1 ± 0.2	3.6 ± 0.2	4.0 ± 0.2
	Female	3.9 ± 0.2	4.1 ± 0.2	3.7 ± 0.2	3.7 ± 0.1
Eye opening[d]	Male	16.8 ± 0.1	16.6 ± 0.2	16.5 ± 0.2	16.1 ± 0.2*
	Female	16.7 ± 0.1	16.6 ± 0.3	16.5 ± 0.2	15.8 ± 0.2**
Age at vaginal opening		36.4 ± 1.1	34.4 ± 0.6	33.9 ± 0.4	34.0 ± 0.6
Age at preputial separation		43.9 ± 0.5	44.0 ± 1.1	44.1 ± 0.5	43.4 ± 0.5

Data are given as mean ± S.E.M. * = significant difference from control, $p < 0.05$; ** $p < 0.01$; $^{\#}$ = significant difference from Aroclor 1254, $p < 0.05$; $^{\#\#}$ $p < 0.01$

[a] anogenital distance (in mm)

[b] crown-rump length (in mm)

[c] age at pinna detachment (in days)

[d] age at bilateral eye opening (in days).

Table 6.2. Organ weights of male and female offspring at PND 4.

Organ	Control	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
PND 4, male	n = 8	n = 0 [a]	n = 6	n = 3
Liver (g)	0.28 ± 0.01		0.30 ± 0.02	0.36 ± 0.02*
Rel. liver weight (%)[b]	3.40 ± 0.06		3.48 ± 0.08$^{\#\#\#\#}$	4.91 ± 0.10****
Kidney (g)	0.11 ± 0.002		0.12 ± 0.006$^{\#}$	0.10 ± 0.006
Rel. kidney weight (%)	1.26 ± 0.03		1.25 ± 0.02	1.23 ± 0.04
Thymus (mg)	22 ± 0.8		24 ± 1.5$^{\#\#\#\#}$	13 ± 0.3****
Rel. thymus weight (%)	0.26 ± 0.01		0.26 ± 0.01$^{\#\#\#}$	0.17 ± 0.01****
PND 4, female	n = 8	n = 8	n = 7	n = 5
Liver (g)	0.29 ± 0.02	0.32 ± 0.01$^{\#}$	0.30 ± 0.02$^{\#}$	0.40 ± 0.03***
Rel. liver weight (%)	3.56 ± 0.23	3.60 ± 0.12$^{\#\#\#\#}$	3.38 ± 0.12$^{\#\#\#\#}$	4.93 ± 0.16****
Kidney (g)	0.11 ± 0.004	0.12 ± 0.004	0.12 ± 0.006	0.11 ± 0.009
Rel. kidney weight (%)	1.28 ± 0.02	1.34 ± 0.03	1.32 ± 0.02	1.35 ± 0.07
Thymus (mg)	21 ± 1.2	24 ± 0.1$^{\#\#}$	22 ± 1.8$^{\#}$	15 ± 0.1**
Rel. thymus weight (%)	0.25 ± 0.01	0.27 ± 0.01	0.25 ± 0.01	0.19 ± 0.01****

Data are given as mean ± S.E.M. * = Significant difference from control, $p < 0.05$; ** $p < 0.01$; ***$p < 0.005$; **** $p < 0.001$; $^{\#}$ = significant difference from Aroclor 1254, $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.005$; $^{\#\#\#\#}$ $p < 0.001$

[a] At PND 4, there were no males left for autopsy after standardization of the litters in this exposure group.

[b] Percentage of total body weight.

F_1 Fertility

The percentage of mated female F_1 offspring (age approximately 260 days) with litters was 64% (corn oil), 88% (0.5 mg/kg 4-OH-CB107), 73% (5.0 mg/kg 4-OH-CB107) and 91% (Aroclor 1254). No effects were observed on the number of matings attempted, the number of resorptions or implantation sites, the number of dead or life fetuses, total litter weight, mean fetal body weight or sexe ratio (data not shown). A slight but not significant increase was observed in the number of corpora lutea (CL) in the left (27%, Figure 6.2A) and right (43%, Figure 6.2B) ovary of pregnant female offspring exposed *in utero* to 0.5 mg/kg 4-OH-CB107. Strikingly, in animals exposed to 5.0 mg/kg 4-OH-CB107 no differences could be observed in the number of CL in the right ovary, whereas in the left ovary this number was increased by 36% compared to control animals ($p = 0.09$). Maternal absolute and relative organ weights (liver, spleen, kidneys, adrenals, thymus) of females offspring at PND 260 exposed *in utero* to 4-OH-CB107 or Aroclor 1254 were not different from corn oil treated females (data not shown). In addition, no effects could be observed on F_2 fetal absolute and relative organ weights (liver, kidney, brain, thymus) at GD20.

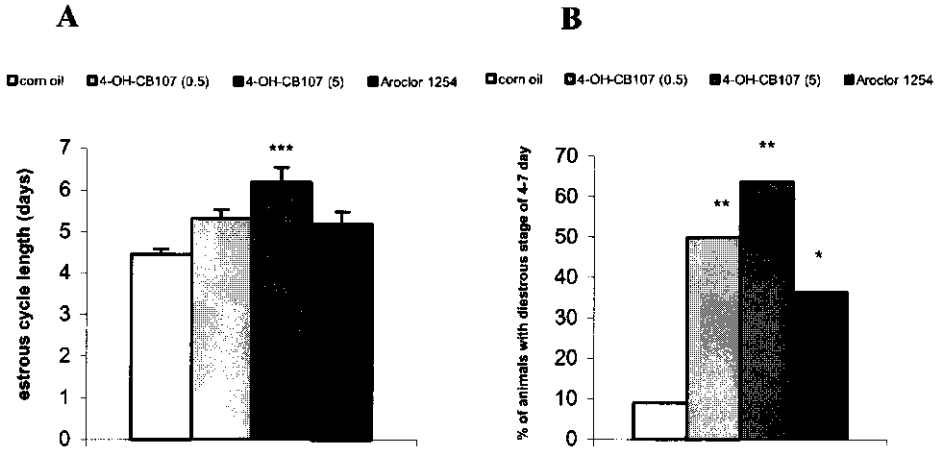

Figure 6.1. Estrous cycle length (A) and percentages of females with a diestrous stage of 4-7 days (B) in offspring, monitored from 210 to 231 days post partum, following in utero exposure to 4-OH-CB107 or Aroclor 1254. Abbreviations used: 4-OH-CB107 (0.5) = offspring exposed *in utero* to 0.5 mg/kg from GD10 to GD16; 4-OH-CB107 (5) = offspring exposed *in utero* to 5 mg/kg 4-OH-CB107 from GD10 to GD16. Statistically significant differences from control are given by * ($p < 0.05$), ** ($p < 0.01$) or **** ($p < 0.001$).

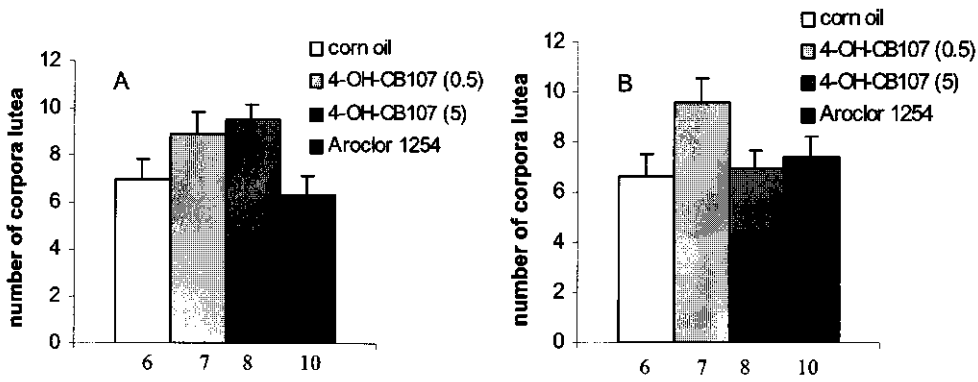

Figure 6.2. Number of corpora lutea in the left (A) and right (B) ovarium of pregnant female offspring at the age of 260 days, following *in utero* exposure to 4-OH-CB107 or Aroclor 1254.

Organ weights of F_1 males and females at 11 months

At 11 months of age, no significant differences were observed in body weights, absolute and relative weights of adrenals, kidneys, liver, spleen, thymus in both male and female offspring exposed *in utero* (data not shown). In addition, male accessory sex organ weights (prostate, testis, seminal vesicle and cauda epididymis) were not affected (Table 6.3). In females from the high 4-OH-CB107 dose

group, the weights of the left ovary were significantly increased by 21% compared to females from the low 4-OH-CB-107 dose group (Table 6.3).

Table 6.3. Sex organ weights of male and female rat offspring (F_1) at PND 325.

Organ	Control	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
Males	n = 11	n = 8	n = 11	n = 11
Prostate	0.42 ± 0.02	0.47 ± 0.02	0.48 ± 0.03	0.39 ± 0.02
Seminal vesicle	1.58 ± 0.06	1.69 ± 0.10	1.61 ± 0.07	1.51 ± 0.07
Testis left	1.76 ± 0.09	1.57 ± 0.18	1.85 ± 0.03	1.81 ± 0.03
Testis right	1.83 ± 0.03	1.59 ± 0.18	1.85 ± 0.04	1.80 ± 0.03
Females	n = 11	n = 8	n = 11	n = 11
Ovarium left (mg)	46 ± 1.1	43 ± 1.2[$]	52 ± 1.9	45 ± 2.6
Ovarium right (mg)	46 ± 2.4	48 ± 2.0	48 ± 1.7	43 ± 1.6
Paired ovarian weight (mg)	92 ± 2.1	91 ± 3.5	99 ± 3.3	88 ± 3.7
Uterus (swollen)	1.43 ± 0.14 (7)	1.10 ± 0.09 (7)	1.36 ± 0.15 (6)	1.06 ± 0.11 (6)
Uterus (not swollen)	0.71 ± 0.05 (7)	0.68 ± 0.06 (4)	0.75 ± 0.05 (6)	0.68 ± 0.04 (10)

Data are given as mean ± S.E.M.
[$] = significantly different from 4-OH-CB107 (5 mg/kg), $p < 0.05$.

Sex steroid hormone levels

Plasma estradiol concentrations of pregnant F_1 offspring (PND 260) showed no significant differences, although levels in animals treated *in utero* with 0.5 mg/kg 4-OH-CB107 showed an increase in estradiol concentrations of approximately 56% (data not shown). Due to high standard deviations, this increase was not significant.

At 11 months of age, plasma estradiol concentrations in female F_1 offspring in the pro-estrous stage, determined by the appearance of a swollen uterus at necropsy, were significantly increased in the 5 mg/kg 4-OH-CB107 treatment group by approximately 230% compared to control animals (Figure 6.3). Estradiol concentrations of female F_1 offspring of which the uterus was not swollen showed no significant differences.

Plasma progesterone levels were unaltered in pregnant F_1 animals (data not shown). In addition, progesterone levels at 11 months of age showed no differences between the exposure groups (Table 6.4). However, estradiol/progesterone ratios were, not significantly, increased in the 5 mg/kg 4-OH-CB107 exposed female offspring in the pro-estrous stage (Table 6.4).

Serum testosterone levels were decreased by 26% (though not significantly) in male offspring at 11 months of age of the 5 mg/kg 4-OH-CB107 dose group (data not shown). Testosterone levels measured in pregnant female F_1 offspring showed no differences between the treatment groups (data not shown).

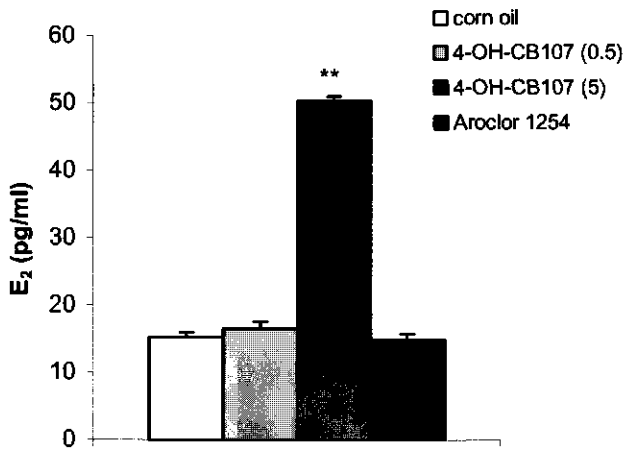

Figure 6.3. Plasma estradiol concentrations in 11 months old female offspring in pro-estrous stage, exposed *in utero* to 4-OH-CB107 or Aroclor 1254. Statistically significant differences from control are given by ** ($p < 0.01$).

Table 6.4. Plasma progesterone (ng/ml) levels and estradiol/progesterone ratios in female offspring at the age of 11 months following in utero exposure to 4-OH-CB107 or Aroclor 1254.

Exposure	Corn oil (2 ml/kg)	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5 mg/kg)	Aroclor 1254 (25 mg/kg)
Proestrous stage (ballooning uterus)				
Progesterone	30.1 ± 4.3	36.6 ± 8.8	36.2 ± 8.9	23.6 ± 2.1
E/P ratio[a)] ($x10^{-3}$)	0.65 ± 0.21	0.50 ± 0.13	1.55 ± 0.5	0.44 ± 0.12
Diestrous stage (no swollen uterus)				
Progesterone	59.5 ± 9.3	47.6 ± 8.2	75.6 ± 8.5	68.5 ± 9.2
E/P ratio	0.25 ± 0.09	0.29 ± 0.08	0.13 ± 0.03	0.12 ± 0.04

The data are presented as mean ± standard error (SEM).
[a)] E/P-ratio = estradiol/progesterone ratio.

Discussion

In the present study the adverse effects caused by *in utero* exposure to a PCB-metabolite were compared with the effect caused by a commercial PCB-mixture (Aroclor 1254). The PCB-metabolite used, 4-hydroxy-2,3,3',4',5-pentaCB (4-OH-CB107), is one of the major OH-PCBs detected in human blood (Bergman *et al.*, 1994) and was shown to accumulate in the blood and brain of fetuses and neonates exposed *in utero* to Aroclor 1254 (Morse *et al.*, 1996a). To our knowledge, this is the first study investigating the possible long-term effects following *in utero* exposure to a PCB-metabolite on development, sex steroid hormone levels and female reproduction.

The most pronounced developmental effect observed following exposure to 4-OH-CB107 was a striking and dose related prolongation of the estrous cycle in female offspring, measured between PND 210-231. This prolongation was also observed in the Aroclor 1254 treated animals, but less pronounced. The total length of the estrous cycle was significantly prolonged in female offspring exposed to 5 mg 4-OH-CB107, indicating that this effect is caused primarily by hydroxylated PCBs instead of parent PCB congeners. Aroclor 1254 and Ah receptor binding PCB congeners have been reported to induce several adverse effects on mammalian endocrine function. For example, prolongation of the estrous cycle (by an increasing length of the diestrous stage) and a delay in the first estrous was observed in female rats after transplacental and translactational exposure to 30 mg/kg Aroclor 1254 for 1 month (Brezner *et al.*, 1984). The increased length of the estrous cycle in female offspring in our study was also a result of a prolonged diestrous stage, determined by the appearance of large amounts of leucocytes in the vaginal smears. A stage representing 11-20 days of diestrous is known as pseudopregnancy (De Feo, 1967). In the present study, the total length of the diestrous stage did not exceed 7 days. In addition, the prolongation of the diestrous stage had no effects on the fertility of the females in this experimental study. However, the disturbances in estrous cycle length may indicate that females exposed *in utero* to especially the PCB-metabolite 4-OH-CB107 may show signs of reproductive senescence at an earlier stage in life compared to corn oil treated females. The first stage of reproductive senescence in rodents is an increase in mean cycle length. Most aging rats then enter a stage of persistent vaginal cornification (PVC), which is often followed by a repetitive pseudopregnancy and finally persistent anestrus (Finch *et al.*, 1984).

Strikingly, plasma estradiol levels in 11-month old female offspring were significantly increased by 230% in the 5 mg/kg 4-OH-CB107 exposed group (in the pro-estrous stage). High plasma E_2 values are often observed in aging rats with a prolonged estrous cycle (Lu *et al*, 1994). It is demonstrated that greater amounts of plasma E_2 in middle aged rats during successive estrous cycles gradually diminishes the neuroendocrine responsiveness to the positive feedback effect of E_2 on LH secretion (LaPolt *et al.*, 1988). Another possible explanation may be the recently published results by Kester *et al.* (2000), who showed that OH-PCBs, including 4-OH-CB107, are extremely potent inhibitors of the human estrogen sulfotransferase (hEST) *in vitro*. In fact, 4-OH-CB107 was one of the strongest of the 32 tested compounds with an IC_{50} of 0.15 – 0.25 nM. Estrogen sulfation is a normal route of reversible inactivation of estradiol. As a result of the inhibition of estrogen sulfation, OH-PCBs may increase the bioavailability of E_2 in target tissues, thereby exerting an indirect estrogenic effect or mimicking the increase in plasma E_2 levels observed in aging female rats.

4-OH-CB107, used as a model PCB-metabolite in this study, is also known to possess anti-estrogenic activity *in vitro* in HeLa cells, or in human T47D breast tumour cells transfected with an estrogen responsive luciferase gene construct (Moore *et al.*, 1997; Meerts, unpublished results). In addition, Moore *et al.* (1997) showed that 4-OH-CB107 significantly displaced [^{3}H]E_2 from the rat uterine cytosolic estrogen receptor, though < 50% displacement was observed at the highest concentration used (10^{-3} M). It is thus very unlikely that the observed increases in E_2 in our study are caused by binding of the metabolite to the estrogen receptor.

Next to the above mentioned effects of 4-OH-CB107 on the estrous cycle length and estradiol concentrations, all other developmental effects observed were caused by the parent compound (Aroclor 1254) only. These effects include a significant increase in the female anogenital distance/crown-rump length ratio (AGD/CRL), an indicator of circulating androgen concentrations over time or of decreased androgen responsiveness. This may indicate a possible partial 'masculinization' of female offspring by Aroclor 1254 treatment. In addition, exposure to 25 mg/kg Aroclor 1254 significantly accelerated eye opening in the offspring by one day. Similar effects have been observed using either TCDD (Gray *et al.*, 1997; Theobald and Peterson, 1997) or Aroclor 1254 (Goldey *et al.*, 1995a). The effect on eye opening is most likely caused by a direct effect of the compound used (i.e. PCBs or TCDD) and not caused by the accompanying hypothyroidism observed in treated offspring, since hypothyroidism is typically associated with a delay in this developmental landmark (Adams *et al.*, 1989; Goldey *et al.*, 1995b). From the present study it can be concluded that accelerated eye opening is most likely an effect of parent PCB congeners, and not their metabolites.

In conclusion, maternal exposure to the PCB-metabolite 4-OH-CB107 results in a significant increase of the estrous cycle length and increased estradiol/progesterone ratios. The effects of the PCB-metabolite are sex-related, since no effects could be detected on male accessory sex organ weights or testosterone levels at postnatal days 310 to 325. The well-known developmental effects of Aroclor 1254 (accelerated eye opening in treated offspring, increased AGD/CRL in female offspring), also shown in this study, could not be observed in offspring exposed to 4-OH-CB107 only. The adverse effects of 4-OH-CB107 on neurotransmitter levels and brain development in rat offspring exposed *in utero* will be published elsewhere (see *Chapter 7*).

Acknowledgements

The authors wish to thank the colleagues from the Toxicology group of the Wageningen University for their assistance at the animal autopsy. We also wish to thank Suzanne Arts, Wilma Blauw, Gerrit van Tintelen, Maria Faassen-Peters and René Bakker for skilful assistance during the animal study. The authors are very grateful to Tatiana Cantillana for synthesis of the PCB-metabolite. This research was financially supported by the European Commission, Environment and Climate Program (ENV4-CT96-0170).

CHAPTER 7

Developmental exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107): Long term effects on brain development, behaviour and brain stem auditory evoked potentials in rats

Abstract

Neurotoxic effects caused by polychlorinated biphenyls (PCBs) have been reported in both humans and animals. The purpose of the present study was to compare the possible developmental neurotoxic effects of a PCB metabolite, 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107), with a mixture of parent PCB congeners (Aroclor 1254), which was found to give rise to an accumulation of mainly 4-OH-CB107 in fetuses following maternal exposure. Pregnant female Wistar WU rats were exposed to 0.5 or 5 mg 4-OH-CB107, or 25 mg Aroclor 1254 per kg body weight from gestation days 10 to 16. Plasma thyroid hormone levels were significantly decreased in male and female offspring of all treatment groups at postnatal day 4 (PND 4). Behavioural experiments using an open field paradigm revealed an impaired habituation in male offspring of all treatment groups at PND 130, whereas no effects in female offspring were observed. Passive avoidance experiments indicated significant influences on the time course of step-down latencies across trials in exposed male rats. Catalepsy induced by the dopamine receptor blocker haloperidol showed increases in latencies to movement onset in female offspring of the low dose (0.5 mg/kg) 4-OH-CB107 exposure group compared to Aroclor 1254 treated offspring at PND 168-175. In contrast, male offspring exposed to 4-OH-CB107, or Aroclor 1254 showed decreases in latencies compared to control animals. Brain stem auditory evoked potentials (BAEPs) measured in male and female offspring at PND 300-310 showed significant increases in auditory thresholds in the low frequency range. In the frequency range from 1 to 4 kHz, peak II latencies in the BAEP were increased in males exposed to 5 mg/kg 4-OH-CB107, but the differences failed to reach statistical significance. A significant treatment-related overall effect was observed on latencies of peak II after click stimulation in both sexes. Male offspring exposed to 4-OH-CB107 exhibited dose-dependent, though not significant prolongations of peak II latency.

Measurements of neurotransmitter levels revealed that developmental exposure to Aroclor 1254 affects both the dopaminergic and serotonergic system, whereas exposure to 4-OH-CB107 affects dopaminergic and noradrenergic systems, with slight but not significant effects on the serotonergic system.

These results indicate that the PCB metabolite 4-OH-CB107 is able to induce long term effects on behaviour and neurodevelopment. The observed effects for 4-OH-CB107 are similar to, but in some aspects different from the effects observed after Aroclor 1254 exposure.

Based on: Meerts I.A.T.M., Lilienthal H., Seegal R.F., Brosch K.O., Hoving S., van den Berg J.H.J., Weijers B.M., Bergman Å, Koeman J.H., and Brouwer A.

Introduction

In utero and lactational exposure to polychlorinated biphenyls (PCBs) may result in developmental effects in the offspring of laboratory animals (reviewed by Brouwer *et al.*, 1995). These developmental effects include alterations in thyroid hormone homeostasis (Morse *et al.*, 1996a; Brucker-Davis 1998; Brouwer *et al.*, 1998), neurobehavioural effects (Tilson *et al.*, 1990; Tilson and Kodavanti, 1997, Schantz *et al.*, 1995, Seo *et al.*, 1995, Lilienthal *et al.* 1997), reproductive and endocrine effects (Peterson *et al.*, 1993, Hany *et al.* 1999a,b) and neurochemical effects (Seegal, 1996; Morse *et al.*, 1996c). The adverse effects caused by PCBs are dependent on the time of exposure and the structural characteristics of the PCB congener. For example, ortho-substituted PCB congeners reduce brain dopamine concentrations in both adult rats and rats exposed in utero through weaning, whereas coplanar, dioxin-like PCB congeners affect neurotransmitter levels predominantly after in utero exposure (Seegal *et al.*, 1996). It is postulated that the changes in neurochemical parameters in PCB-exposed offspring may be causatively linked to the observed neurobehavioural changes such as locomotor activity, delayed spatial learning, and active and passive avoidance (Seegal *et al.*, 1996; Schantz *et al.*, 1995; Seo *et al.*, 1995). The mechanism by which PCBs interfere with neuronal development causing long term effects on neurobehaviour is unknown, but PCB-induced hypothyroidism (reviewed in Brouwer *et al.*, 1998) as well as the observed changes in neurotransmitter levels are suggested to play a role. Perinatal exposure to Aroclor 1254 is known to reduce fetal and neonatal thyroid hormone levels in rats (Morse *et al.*, 1996a). Long term effects observed in offspring exposed perinatally to Aroclor 1254 are e.g. alterations in serotonin metabolism in several brain areas (Morse *et al.*, 1996c) and a selective low-frequency hearing loss (Goldey *et al.*, 1995). Goldey and Crofton (1998) showed that this hearing loss could be partially prevented by T_4 replacement.

Previous studies at our laboratory have shown that exposure of pregnant rats to Aroclor 1254 from gestation days 10 to 16 resulted in a substantial accumulation of mainly one hydroxylated metabolite (2,3,3',4',5-pentachlorobiphenyl, 4-OH-CB107) in the fetal compartment, especially in the brain (Morse *et al.*, 1996a). This PCB metabolite is one of the major metabolites identified in blood samples of seals, rats and humans (Bergman *et al.*, 1994, Sjödin *et al.*, 1999). 4-OH-CB107 is a metabolite of 2,3,3',4,4'-pentachlorobiphenyl (CB-105) and of 2,3',4,4',5-pentachlorobiphenyl (CB-118) (Sjödin *et al.*, 1998). The presence of 4-OH-CB107 in blood plasma of humans and wildlife, its observed accumulation in brain from animals exposed to Aroclor 1254, and its potency to induce drastic decreases in thyroid hormone levels following prenatal exposure (Meerts *et al.*; submitted,

Chapter 6), prompted us to investigate the potency of 4-OH-CB107 to induce possible neurobehavioural and neurochemical changes in rat offspring exposed *in utero*. To compare the effects observed by 4-OH-CB107 with effects caused by parent PCBs, one group of animals was dosed with Aroclor 1254.

Animals, Materials and Methods

Chemicals

4-Hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) was synthesized as described by Bergman *et al.* (1995) and at least 99.9% pure. Aroclor 1254 was kindly donated by Prof. Dr. M. van den Berg (Ritox, University of Utrecht, The Netherlands).

Animals and treatment

All experimental procedures involving animals were approved by the Animal Welfare Committee of Wageningen University. Wistar WU rats (60 females, 30 males; 14 weeks old) were purchased from Charles River (Sulzfeld, Germany) and allowed to acclimatize for three weeks. The rats were maintained in macrolon cages in rooms with 50 ± 10% humidity and 21 ± 2°C, in an artificial 12-h:12-h light-dark cycle with lights on at 06:00 h. Rat chow (Hope Farms, Woerden, the Netherlands) and tap water were supplied *ad libitum*. After the acclimatization period two females were placed in a cage with one male overnight from 17:00 to 8:00 hr. Copulation was examined each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, this day was designated as day 0 of gestation (GD0) and females were housed individually. On day 10 of gestation the pregnant rats were divided randomly into the different treatment groups and transferred to a macrolon, stainless steel cage to facilitate the collection of PCB-contaminated faeces.

Pregnant females (13 per exposure group) received a daily oral dose of 0, 0.5 or 5 mg 4-OH-CB107 per kg body weight dissolved in corn oil (2 ml/kg body weight) from gestation days 10 to 16. For comparison of the effects of the PCB-metabolite with effects caused by parent PCB congeners, a fourth group of rats was dosed with 25 mg Aroclor 1254 per kg body weight from gestation days 10 to 16. This dose level was chosen since 25 mg/kg Aroclor 1254 given to pregnant rats gave rise to a PCB metabolite production equivalent to 5 mg/kg of 4-OH-CB107 (Morse *et al.*, 1996a). Maternal body weights were monitored daily throughout gestation and lactation. On day 20 of gestation, pregnant females were transferred to bedding material and were given paper tissues to make a litter. The offspring were counted, inspected for signs of overt toxicity and weighed at birth (PND 0), PND1, 4, 7, 14 and 21. After weaning, body weights of the offspring were monitored weekly until sacrifice. On PND 4, litters were standardized to 4 males and 4 females. Generally, this required culling of excess offspring. However, in a few cases the standardized litter required pups from two dams. To maintain litter independence, no dam was allowed to contribute pups to more than one litter. In addition, pups transferred from one litter to another were not used for any analysis. The standardized litter became the experimental unit and all treatment mean values reported are litter based. The pups were numerically marked on their feet to identify individual animals within a litter.

Excess pups were decapitated at PND4 and trunk blood was collected for thyroid hormone analysis. Liver, kidneys, brain and thymus were weighed.

Following weaning at PND 21, pups were housed with littermates in unisexual groups, two pups per cage. After puberty, the offspring was split into two cohorts. One cohort (n = 41 litters with 2 males and 2 females per litter) was used for examining reproductive effects (described in Meerts *et al.*, submitted; *Chapter 6*). Animals from the other cohort (also 41 litters with 2 males and 2 females per litter) were housed in unisexual groups. At least two weeks before the onset of behavioural testing, animals were housed individually in unisexual groups.

Thyroid hormone analysis

Plasma total T_4 (TT_4), free T_4 (FT_4) and total T_3 (TT_3) were analysed in duplicate using chemiluminescence kits and plasma thyroid stimulating hormone (TSH) concentrations were analysed with a specific rat TSH immunoassay. All kits were purchased from Amersham (Amersham, Buckinghamshire, UK). The intra- and interassay variations were below 10% for all hormones.

Behavioural testing

Behavioural tests were conducted in naive male and female offspring. Only one randomly selected male and female rat per litter was used for one behavioural test. The experiments were performed in a blind fashion.

At PND 130, locomotor activity of male and female offspring was measured in an open field paradigm following the procedures described in Hany *et al.* (1999a). Briefly, 8 males and 8 females per exposure group (from different litters) were placed in a white octagonal arena with a diameter of 75 cm for 9 minutes, subdivided into three intervals of three minutes each. The open field was evenly illuminated by indirect light provided by two lamps (40 W each). The movements of the animals were recorded by a video camera, which was connected to a digital image processing system (Ethovision, Noldus, Wageningen, The Netherlands). The plane of the open field was subdivided in an inner zone, measuring 50 cm in diameter, and an outer zone, consisting of the remaining outer ring.

The passive avoidance behaviour was studied in a step down task at PND130 as described in detail by Weinand-Härer *et al.* (1997). In short, a 1 mA footshock with a 1-s duration was used in the conditioning trial. Subsequently, step-down latencies from a platform were tested 5 minutes, 4 hours and 24 hours after the conditioning, with a maximal duration of 180 s per trial.

Between PND 168 and 175, catalepsy induced by the dopamine receptor blocker haloperidol was tested in male and female offspring as described by Weinand-Härer *et al.* (1997). Only females in the diestrous stage of the estrous cycle were used for the test. Haloperidol was injected intraperitoneally at a concentration of 0.3 mg/kg body weight, and the animals were tested 30 min and 60 min after injections by placing the rat in three postures: (i) placing both front paws on a horizontal bar 9 cm above the surface, (ii) putting the rat on a grid, with a 10 degrees deviation from the vertical plane, and (iii) placing the paws in four different holes of a box. Time for retraction of the first paw and descent latency, latency to movement onset, and retraction of a front leg and a hind leg

were determined on conditions (i), (ii), and (iii), respectively. If the rat failed to move one paw, testing was terminated at 180s on all conditions.

Brain stem auditory evoked potentials

Between PND300 to 310 auditory thresholds and peak latencies were studied in male and female offspring by recording brain stem auditory evoked potentials (BAEPs) using methods adapted from Lilienthal and Winneke (1996). The animals were housed individually during two weeks before the start of the BAEP measurements. The animals were sedated with an i.p. injection of ketamine (90 mg/kg body weight for males, 55 mg/kg body weight for females) and maintained on xylazine (3.5 mg/per kg body weight for males, and 3 mg/kg body weight for females). Rats were placed on a heating pad to prevent from cooling. Needle electrodes were placed under the skin at the vertex and behind both ears. The ground electrode was contralateral to the stimulated right ear. The left ear was occluded by a tissue plug in the outer ear channel. Impedance was 5 kΩ at the maximum.

Brain stem auditory evoked potentials were recorded on a Pathfinder II (Nicolet Inc., Madison, WI) after stimulation with rarefaction clicks using a shielded high frequency piezo loudspeaker and a SM 700 multisignal auditory generator. Clicks were presented at seven different sound pressure levels (72, 62, 52, 42, 32, 22, and 12 dB, re. 20 μPa) using a repetition rate of 11.1 Hz. The pulse width was set to 50 μs. In addition, BAEPs evoked by tone pips at different frequencies (20, 16, 8, 4, 2, 1, and 0.5 kHz) were recorded at sound pressure levels (SPL) ranging from 88 to 8 dB. Because of the general lower hearing capacity at the lower frequency border in rats, higher sound pressure levels, up to 110 dB, were used at 0.5 kHz for threshold determination. Tone pips with frequencies below 4 kHz were delivered by shielded TDH 39P earphones. Sound pressure levels were calibrated with a precision sound level meter (type 2230, Brüel & Kjaer) equipped with a 0.5'' condenser microphone (type 4165, Brüel & Kjaer). The whole set-up was calibrated using a pistonphon (type 4220, Brüel & Kjaer).

For recording, the sweep duration was set at 8 ms. Sweeps were sampled with a rate of 62.5 kHz. For each BAEP 1000 sweeps were averaged using the artefact rejection. For the determination of thresholds, sound pressure level was progressively lowered until even the most prominent peak No. II was no longer identified in the BAEP.

Dissections

Dams were sacrificed at weaning (PND21) under ether anaesthesia and blood was collected via the *vena cava* in heparinized tubes for thyroid hormone measurements. Liver, kidneys, adrenals, thymus, brain, spleen, uterus and ovary were collected, blotted dry and weighed.

Male and female offspring were sacrificed between postnatal days 310 to 325. The animals were killed by decapitation within 15 s of removal from their cages. Dissections were conducted between 08:00 and 12:00. Trunk blood was collected in Eppendorf tubes (for serum preparation) and heparinized tubes (for plasma) for hormone analysis. Brains were dissected rapidly on ice (within 5 minutes) and separated into the following regions: lateral olfactory tract (LOT), prefrontal cortex (PFC), frontal cortex (FC), caudate nucleus (CN), and nucleus accumbens (NA). Brain regions were weighed, immediately frozen in liquid nitrogen and stored at –80°C until analysis of biogenic amines.

Measurement of biogenic amines

Brain regions were thawed by the addition of 10 volumes of ice cold 0.2N perchloric acid containing 100 mg/l of ethylene glycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and homogenized on ice with an ultrasonic tissue disruptor (Vibra cell, Sonics& Materials Inc. Danbury, CT, USA) for 30 seconds. The regional brain levels of the neurotransmitters dopamine (DA), 5-hydroxytryptamine (5-HT), and norepinephrine (NE) as well as the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the 5-HT metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by HPLC separation and electrochemical analysis as described before (Seegal *et al.*, 1986).

Statistical analysis

Statistical analyses were performed using the SPSS or SAS statistical software package. Depending on the data structure different statistical analyses were conducted. The data of the open-field and passive avoidance test were assessed by analysis of variance (ANOVA) with repeated measures on the factor time. Wilk's Lambda was used to analyze within subjects effects. The catalepsy data were analyzed with the median test (Siegel, 1952). BAEP results were analyzed using multivariate analysis of variance (MANOVA) with repeated measures on the factors SPL and rate. Sex was included as an independent factor together with treatment in the multivariate analysis. In addition, preplanned univariate ANOVA's with repeated measures on one factor, SPL or rate, were calculated and separate ANOVA's were conducted for each gender. Following significant overall F-tests post hoc comparison of group means was performed using the Ryan-Einot-Gabriel-Welsch multiple range test.

Data on regional brain biogenic amine levels and thyroid hormone levels were evaluated with one-way analysis of variance (ANOVA). Levene's test was used to evaluate homogeneity of variances, and the Bonferroni test was used to compare individual treatment means when ANOVA indicated that significant differences were present. When the Levene's test was significant, a log transformation of the data was performed prior to ANOVA.

Results

Body and organ weights

No effects were observed on maternal body weight gain, mean and total fetal body weight, number of implantation sites, resorptions, number of fetuses per litter, or sex ratio following prenatal exposure to 4-OH-CB107 or Aroclor 1254 from gestation days 10 to 16 (data not shown). Developmental landmarks (i.e. pinna detachment, age at eye opening, anogenital distance, crown rump length, age at vaginal opening and preputial separation), estrous cyclicity and F_1 reproduction effects observed following 4-OH-CB107 or Aroclor 1254 exposure are reported elsewhere (Meerts *et al.*, submitted; *Chapter 6*).

Plasma thyroid hormone levels

Plasma thyroid hormone and thyroid stimulating hormone (TSH) levels from 4-OH-CB107 or Aroclor 1254 exposed dams showed no significant differences relative to controls at 21 days postpartum (data

not shown). However, four days after birth (PND 4), male and female neonatal total thyroxine (TT_4) levels were significantly decreased in all exposure groups (Figure 7.1A). The decrease in TT_4 levels was highest in the Aroclor 1254 exposed group (66% and 42% decrease in male and female offspring, respectively, compared to control animals). Plasma FT_4 levels at PND 4 showed a dose dependent, though not significant, reduction in 4-OH-CB107 exposed male offspring compared to controls (Figure 7.1B). Aroclor 1254 exposed male offspring also showed a decrease in FT_4 levels, but this was not significant possibly because of the low number of animals in this group (N = 6). In female offspring, the effects on FT_4 levels were less pronounced (Figure 7.1B).

Plasma TT_3 levels at PND 4 were decreased in both male and female offspring of the Aroclor 1254 exposed animals, but this reduction was only significant in males (Figure 7.2). No decreases in TT_3 levels were observed in offspring exposed to 4-OH-CB107. In addition, neonatal TSH levels were not affected at PND 4 in both male and female offspring of the different treatment groups (data not shown).

At 11 months of age, no differences could be observed in thyroid hormone or TSH levels in male and female offspring (data not shown).

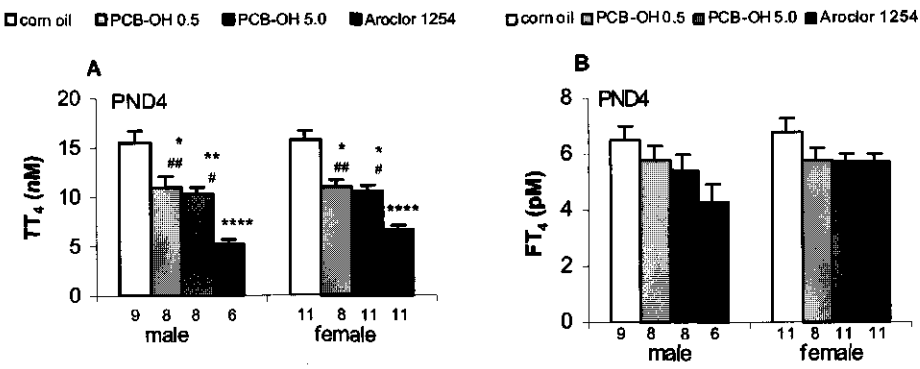

Figure 7.1. Total thyroxine (TT_4, A) and free thyroxine (FT_4, B) levels in male and female offspring at postnatal day 4 (PND 4) following maternal exposure to corn oil, 4-OH-CB107 (PCB-OH) or Aroclor 1254. $^{\#}$ Significant differences from Aroclor 1254 ($p < 0.05$); $^{\#\#}$ ($p < 0.01$);.* Significant differences from control ($p < 0.05$); ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$. The number of different litters is given at the base of each column.

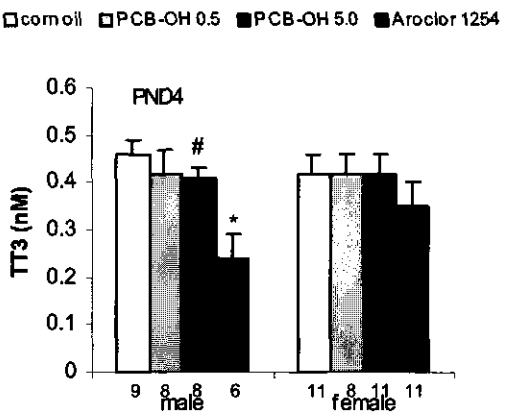

Figure 7.2. Total triiodothyronine (TT_3) levels in male and female offspring at postnatal day 4 (PND 4) following maternal exposure to corn oil, 4-OH-CB107 (PCB-OH) or Aroclor 1254. * = significantly different from control ($p < 0.05$); # = significantly different from Aroclor 1254 ($p < 0.05$). The number of different litters is given at the base of each column.

Behavioural tests

Locomotor activity

In male offspring, all exposed groups showed a significantly higher overall locomotor activity in the open field paradigm in the last 3 minutes compared to controls (Fig. 7.3A). There was a significant interaction between exposure and time [$F(6,52)=3.37$; $p < 0.05$] as well as a significant quadratic contrast for exposure [$F(3,27)=3.52$; $p < 0.05$], indicating a different time course of activity in different treatment groups. Post hoc testing revealed significant elevations of locomotor activity in all exposed groups in comparison to controls during the last 3 minutes of the measuring period ($p < 0.05$). No effects could be seen in female offspring (Figure 7.3B). There was also no exposure-related difference in the preference for the outer or inner zone in both sexes (data not shown).

Passive avoidance

There were no exposure-related differences in avoidance latencies in female rats at PND 130. Data indicated a reduction of latencies 4 hours after the conditioning trial in male rats of the low dose 4-OH-CB107 group, but not in the high dose group (Figure 7.4A). According to ANOVA with repeated measures, there was a significant interaction between exposure and time [$F(6,52)=2.24$; $p \leq 0.05$] and a significant quadratic contrast, illustrating exposure-related differences in the course of latencies across the trials [$F(3,27)=4.98$; $p<0.05$]. In addition, the reduction of latencies in the low dose 4-OH-CB107 group was significant in comparison to controls and the high dose group 4 h after the conditioning trial according to post hoc testing ($p < 0.05$). To verify these subtle changes, the measurements were repeated by testing naive littermates at PND 290 (Figure 7.4B).

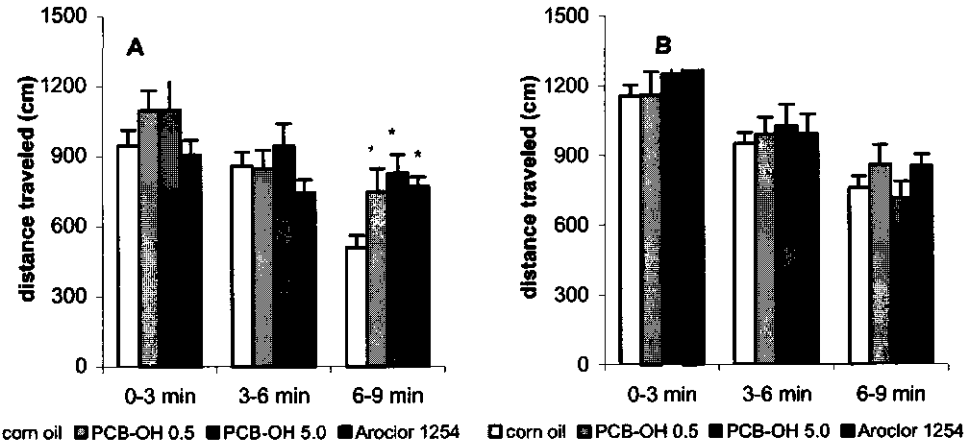

Figure 7.3. Total distance (in cm) travelled by male (A) and female (B) rats in the whole arena of the open field. * = Significantly different from control ($p < 0.05$). PCB-OH = 4-OH-CB107. Total number of litters per exposure group was eight, except for male offspring exposed to 0.5 mg/kg 4-OH-CB107 (N = 7).

Again, a significant interaction between time and exposure was detected [$F(6,54)=2.47$; $p < 0.05$] as well as a significant linear contrast [$F(3,28)=2.97$; $p < 0.05$]. A steady increase in latencies across the trials could be observed only in the Aroclor 1254 group, whereas a plateau was reached in all other groups. These differences could not be attributed to different treatment groups according to post hoc tests.

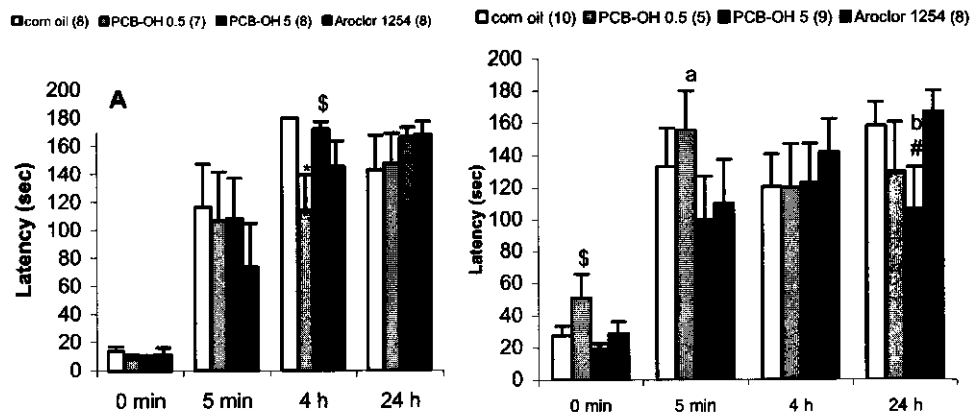

Figure 7.4. Latencies (in sec) in the passive avoidance task from naive males at PND 130 (A) and PND 290 (B). Between brackets the number of litters per exposure group is given. a = marginally different from 4-OH-CB107 5 mg/kg; $p < 0.1$; b = marginally different from corn oil; $p < 0.1$; # = significantly different from Aroclor 1254; $p < 0.05$; $ = significantly different from 4-OH-CB107 5 mg/kg; $p < 0.05$. Abbreviations used: PCB-OH = 4-OH-CB107.

Catalepsy

In males, significant differences between groups were detected in the latency to move a front paw on the grid (Figure 7.5A,B). Sixty minutes after haloperidol injection, the time needed to move the front paw was significantly reduced in rats treated with the low dose of 4-OH-CB107 (0.5 mg/kg; $p < 0.05$) and rats treated with Aroclor 1254 ($p < 0.01$) compared to controls. The values of the high dose group of 4-OH-CB107 were changed in the same direction, though not significantly. No significant treatment related effects were detected in males on the bar or the box.

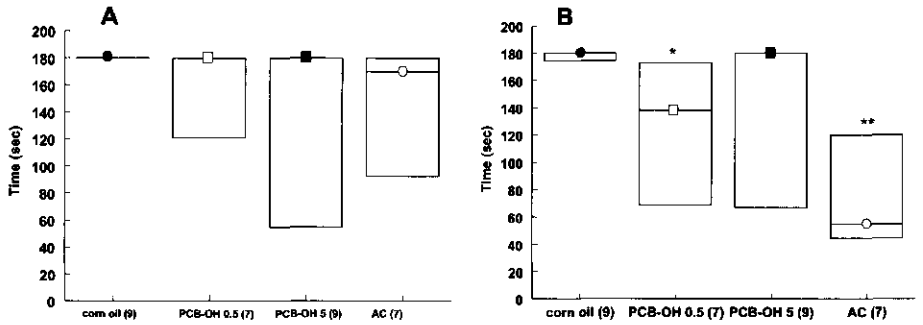

Figure 7.5. Latency to move a front paw on the grid in males, 30 minutes (A) and 60 minutes (B) after the challenge with haloperidol. Median, 25% and 75% quartiles are presented. Between brackets the number of litters per exposure group is given. Significant difference to control group: * $p < 0.05$; ** $p < 0.01$. Abbreviations used: PCB-OH = 4-OH-CB107; AC = Aroclor 1254.

In females, a marginally significant increase ($p < 0.1$) was observed in the latency to remove a front paw on the grid in the low dose 4-OH-CB107 group compared to controls 30 minutes after haloperidol injection (Figure 7.6A). In Aroclor 1254 treated females, this increase was more pronounced ($p < 0.05$). Sixty minutes after haloperidol injection, the latency was marginally reduced ($p < 0.1$) in the low dose 4-OH-CB107 group compared to the high dose group (Figure 7.6B). Latencies to retract a front leg or a hind leg from the box in females 30 minutes after haloperidol injection were significantly increased in the low dose PCB-OH group compared to Aroclor treated females (data not shown).

Brain stem auditory evoked potentials

Auditory thresholds

For the tone pips, there were significant treatment-related influences on auditory thresholds at 0.5 kHz [$F(3,56)=4.64$; $p < 0.05$] and 2 kHz [$F(3,56)=2.87$; $p < 0.05$] in a two-way ANOVA with gender and treatment as independent factors. Gender exerted a significant effect at 0.5 kHz ($p < 0.05$), but not at 2 kHz ($p < 0.1$).

In females, auditory thresholds were significantly affected by exposure at 1 kHz [$F(3,34)=3.39$; $p< 0.05$)]. Thresholds were elevated in Aroclor 1254 exposed females compared to all other groups, the mean increases to controls measuring 1.7, 4.7 and 1.7 dB at 0.5, 1, and 2 kHz,

respectively, but statistical significance (post hoc test, $p < 0.05$) was obtained only for the difference between Aroclor 1254 treated females and female rats exposed to 5 mg/kg 4-OH-CB107 (Figure 7.7). This outcome was supported by a significant interaction between frequency and treatment [$F(18,204) = 2.27$; $p < 0.01$] across all frequencies. There were no significant auditory threshold deficits in males (data not shown), only a marginally significant treatment effect at 500 Hz [$F(3,22) = 2.60$; $p < 0.08$].

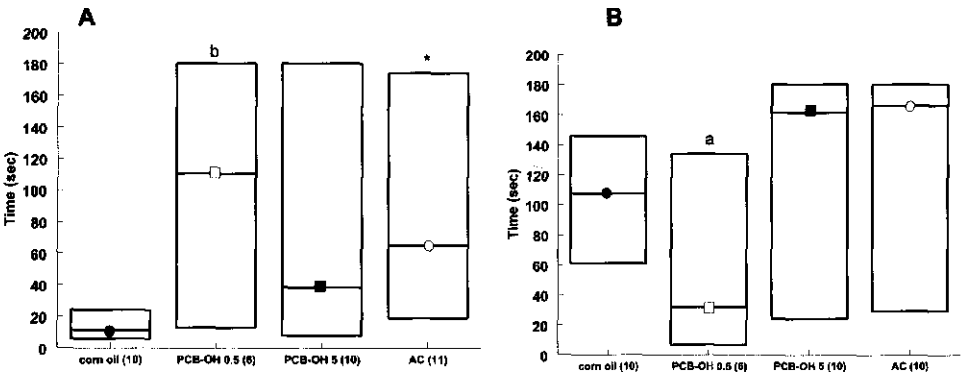

Figure 7.6. Latency to move a front paw on the grid in females, 30 minutes (A) and 60 minutes (B) after the challenge with haloperidol. Median, 25% and 75% quartiles are presented. Between brackets the number of litters per exposure group are given. a = marginally different from 4-OH-CB107 (5 mg/kg); $p < 0.1$; b = marginally different from corn oil; $p < 0.1$; * = significantly different from corn oil, $p < 0.05$. Abbreviations used: PCB-OH = 4-OH-CB107; AC = Aroclor 1254.

With the exception of a significant overall gender effect [$F(1,58) = 4.44$; $p < 0.05$], no exposure related effects were detected on click thresholds.

Peak latencies

Analysis of peak II at different frequencies and sound pressure levels (SPL) revealed a significant interaction between SPL and treatment at the 500 Hz frequency in males [$F(12,84) = 2.43$; $p < 0.05$, data not shown]. Representative traces of BAEPs at 1 kHz and 88 dB are shown in figure 7.8. Latency values of peak II at 1 kHz are given for three different sound pressure levels in Table 7.1. Compared to all other groups, male animals treated with 5 mg/kg 4-OH-CB107 exhibited the highest latency values at all frequencies and SPL tested. In addition, 4-OH-CB107-induced prolongation of peak II latency was dose-dependent at all levels used at 1 kHz. However, differences failed to reach statistical significance. Similar results were obtained for 2 kHz and 4 kHz (data not shown).

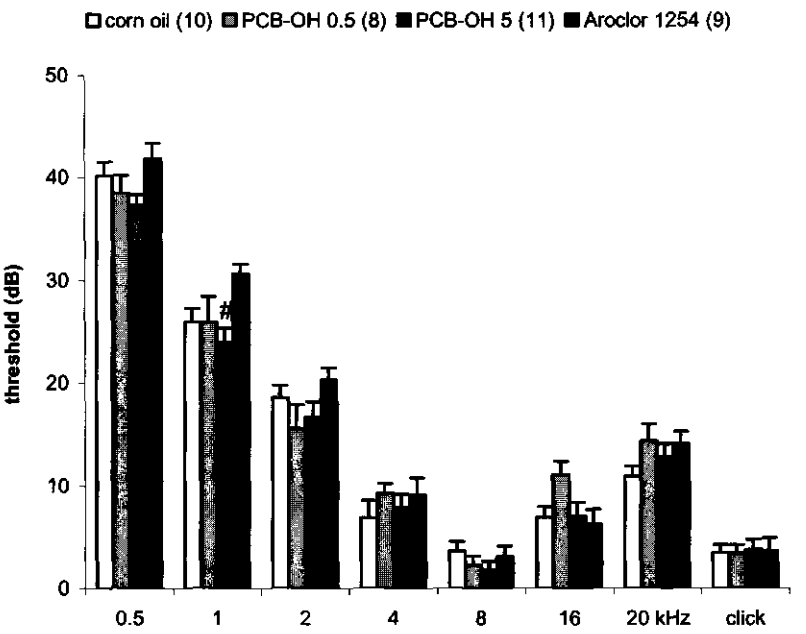

Figure 7.7. Auditory thresholds at different tone frequencies in female offspring at PND 300-310 following maternal exposure to corn oil, 4-OH-CB107 (PCB-OH) or Aroclor 1254. # = Significantly different from Aroclor 1254, $p < 0.05$. The number of animals from different litters used per treatment group is given between brackets in the legend.

After click stimulation using different SPL, there was a significant main effect for exposure on peak II latency in both sexes [$F(3,54)=3.06$; $p < 0.05$]. According to post hoc tests, there were no significant differences between genders. Latency values for peak II are shown in Table 7.1 (for 1 kHz) and 7.2 (for clicks). Male rats exposed to 4-OH-CB107 exhibited dose-dependent increases in peak II latencies on all, but the lowest SPL in comparison to controls and Aroclor 1254 treated rats. Representative BAEP traces for clicks at 72 dB are shown in Figure 7.9. According to post hoc tests, there was a significant difference between males exposed to the low dose of 4-OH-CB107 and Aroclor 1254 treated animals at the lowest SPL ($p < 0.05$). All other differences were not significant.

In females dose-dependent increases in latencies due to metabolite exposure were observed only at the three lowest SPLs, but there were no significant differences between groups. Also, no significant differences were observed on latencies of peak IV and the interpeak latency between II and IV (data not shown).

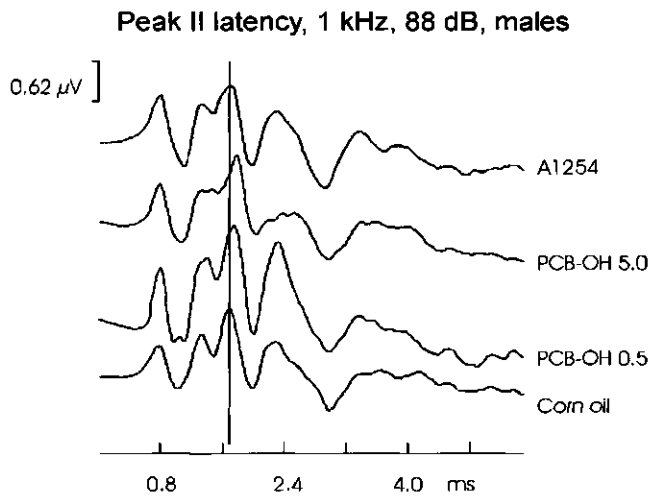

Figure 7.8. Grand averages for BAEPs at 1 kHz and 88 dB SPL, abscissa 0.8 ms/div, ordinate 0.620 μV/div, groups from bottom to top, corn oil, PCB-OH 0.5 (=4-OH-CB107; 0.5 mg/kg bw), PCB-OH 5.0 (=4-OH-CB107; 5 mg/kg bw), A1254 (= Aroclor 1254). The stimulus artefact was removed from the traces.

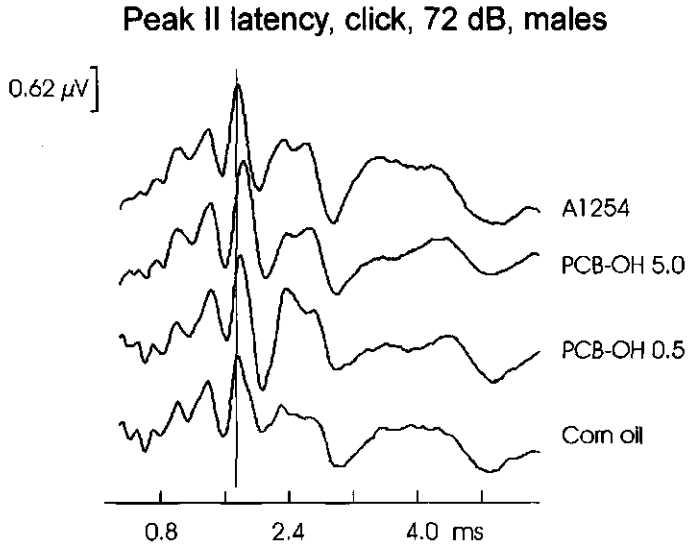

Figure 7.9. Grand averages for BAEPs after click stimulation at 72 dB SPL, abscissa 0.8 ms/div, ordinate 0.620 μV/div, groups from bottom to top, corn oil, PCB-OH 0.5 (=4-OH-CB107; 0.5 mg/kg bw), PCB-OH 5.0 (=4-OH-CB107; 5 mg/kg bw), A1254 (= Aroclor 1254). The stimulus artefact was removed from the traces.

Table 7.1. Latency of peak II at different frequencies and sound pressure levels (SPL)

Exposure	1 kHz		
SPL (dB)	48	68	88
Female offspring			
Corn oil	1.987 ± 0.047	1.818 ± 0.032	1.774 ± 0.044
4-OH-CB107 (0.5)	2.087 ± 0.049	1.918 ± 0.042	1.881 ± 0.065
4-OH-CB107 (5)	2.040 ± 0.035	1.838 ± 0.024	1.806 ± 0.043
Aroclor 1254	2.040 ± 0.037	1.866 ± 0.033	1.768 ± 0.025
Male offspring			
Corn oil	1.961 ± 0.028	1.803 ± 0.025	1.737 ± 0.041
4-OH-CB107 (0.5)	1.995 ± 0.047	1.840 ± 0.024	1.739 ± 0.046
4-OH-CB107 (5)	2.088 ± 0.125	1.934 ± 0.115	1.818 ± 0.146
Aroclor 1254	1.986 ± 0.027	1.778 ± 0.020	1.646 ± 0.059

Data are presented as mean ± SE. $^{\#}$ = significantly different from Aroclor, $p < 0.05$. Abbreviation used: PCB-OH = 4-OH-CB107, SPL = sound pressure level.

Biogenic amines

3,4-Dihydroxyphenylacetic acid (DOPAC) levels were significantly decreased by 37% ($p < 0.05$) in the nucleus accumbens (NA) of male offspring exposed to 5 mg/kg 4-OH-CB107 compared to control animals (Table 7.3).

In the caudate nucleus (CN), DOPAC levels were significantly lower in the 5 mg/kg 4-OH-CB107 group compared to Aroclor 1254 ($p < 0.01$), but not compared to controls. In females, a slight though not significant increase in DOPAC levels was observed in 4-OH-CB107 and Aroclor 1254 treated animals compared to controls in the NA, and a slight but not significant decrease in DOPAC levels in the CN of the 5 mg/kg 4-OH-CB107 dose group.

5-Hydroxyindole-3-acetic acid (5-HIAA) levels were significantly increased in the frontal cortex (FC) of Aroclor 1254 treated male offspring compared to controls (by 62.5%) and 4-OH-CB107 (by 50%) treated animals. The same trend, though not significant, is visible in female offspring. In the caudate nucleus (CN) 5-HIAA levels were significantly increased by 50% ($p < 0.01$) in males from the low 4-OH-CB107 group compared to Aroclor 1254 treated animals.

The concentrations of homovanillic acid (HVA) in the CN of male animals were significantly decreased by 22.5% ($p < 0.05$) in the high 4-OH-CB107 group compared to controls. No effects were observed on the ratio of DOPAC/DA and 5-HIAA/5-HT in different brain regions in both male and female offspring.

Table 7.2. Latency of peak II at different frequencies and sound pressure levels (SPL)

Exposure	Click						
SPL (dB)	**12**	**22**	**32**	**42**	**52**	**62**	**72**
Female offspring							
Corn oil	2.147 ± 0.017	2.000 ± 0.017	1.925 ± 0.018	1.874 ± 0.017	1.829 ± 0.020	1.832 ± 0.020	1.770 ± 0.024
4-OH-CB107 (0.5)	2.222 ± 0.031	2.070 ± 0.029	1.944 ± 0.021	1.914 ± 0.028	1.864 ± 0.024	1.864 ± 0.023	1.814 ± 0.025
4-OH-CB107 (5)	2.228 ± 0.070	2.118 ± 0.066	2.004 ± 0.071	1.901 ± 0.023	1.850 ± 0.022	1.850 ± 0.024	1.792 ± 0.018
Aroclor 1254	2.144 ± 0.021	2.032 ± 0.027	1.913 ± 0.024	1.876 ± 0.026	1.804 ± 0.021	1.813 ± 0.020	1.769 ± 0.014
Male offspring							
Corn oil	2.163 ± 0.039	2.029 ± 0.034	1.904 ± 0.026	1.872 ± 0.034	1.819 ± 0.030	1.840 ± 0.033	1.773 ± 0.028
4-OH-CB107 (0.5)	2.267$^{\#}$ ± 0.046	2.037 ± 0.037	1.915 ± 0.030	1.878 ± 0.035	1.856 ± 0.056	1.861 ± 0.044	1.819 ± 0.072
4-OH-CB107 (5)	2.178 ± 0.037	2.150 ± 0.111	2.046 ± 0.098	1.994 ± 0.094	1.964 ± 0.099	1.978 ± 0.094	1.908 ± 0.099
Aroclor 1254	2.125 ± 0.027	1.986 ± 0.020	1.897 ± 0.015	1.845 ± 0.016	1.790 ± 0.021	1.790 ± 0.021	1.730 ± 0.028

Data are presented as mean ± SE. $^{\#}$ = Significantly different from Aroclor 1254, p, < 0.05. Abbreviation used: SPL = sound pressure level.

Table 7.3. Biogenic amine concentrations (ng/mg tissue, fresh weight) in different brain regions of male and female offspring at the age of 11 months, following *in utero* exposure to 4-OH-CB107 or Aroclor 1254 from gestation days 10 to 16.

	Brain region	Control	4-OH-pentaCB (0.5 mg/kg)	4-OH-pentaCB (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
Male					
DOPAC	NA	2.63 ± 0.19 (10)	2.25 ± 0.37 (7)	1.66 ± 0.21* (10)	2.50 ± 0.27 (8)
DOPAC	CN	2.27 ± 0.10 (11)	2.24 ± 0.23 (7)	1.86 ± 0.14$^{\#\#}$ (11)	2.70 ± 0.21 (8)
5-HIAA	FC	0.024 ± 0.002 (11)	0.023 ± 0.002$^{\#\#}$ (6)	0.026 ± 0.002$^{\#}$ (10)	0.039 ± 0.004** (8)
5-HIAA	CN	0.020 ± 0.001 (11)	0.027 ± 0.003$^{\#\#}$ (7)	0.021 ± 0.001 (10)	0.018 ± 0.002 (8)
HT	FC	0.012 ± 0.001 (11)	0.011 ± 0.001$^{\#}$ (6)	0.014 ± 0.001 (10)	0.021 ± 0.004* (8)
HVA	PFC	0.033 ± 0.006 (9)	0.043 ± 0.007 (4)	0.024 ± 0.004 (6)	0.023 ± 0.004 (6)
HVA	CN	0.71 ± 0.02 (10)	0.63 ± 0.06 (7)	0.55 ± 0.03* (11)	0.66 ± 0.04 (8)
DOPAC/ DA (%)	CN	16.0 ± 1.0 (10)	20.7 ± 1.6 (7)	16.7 ± 0.9$^{\#}$ (11)	22.2 ± 2.0* (8)
Female					
DOPAC	NA	3.20 ± 0.34 (8)	4.21 ± 0.23 (7)	4.16 ± 0.39 (10)	4.32 ± 0.33 (10)
DOPAC	CN	3.25 ± 0.37 (9)	3.41 ± 0.42 (8)	2.83 ± 0.21 (10)	3.76 ± 0.43 (10)
5-HIAA	FC	0.037 ± 0.003 (9)	0.039 ± 0.003 (8)	0.037 ± 0.003 (9)	0.045 ± 0.003 (9)
5-HIAA	CN	0.025 ± 0.001 (9)	0.031 ± 0.001 (7)	0.029 ± 0.002 (9)	0.029 ± 0.002 (9)
HVA	PFC	0.051 ± 0.009 (9)	0.077 ± 0.014 (8)$^{\#}$	0.049 ± 0.005 (9)	0.034 ± 0.005 (10)
HVA	CN	0.73 ± 0.05 (9)	0.76 ± 0.10 (8)	0.61 ± 0.04 (10)	0.67 ± 0.07 (10)
DOPAC/ DA (%)	CN	36.4 ± 5.7 (9)	41.2 ± 8.1 (8)	29.0 ± 4.0 (9)	46.0 ± 6.4 (10)

Data are given as mean ± S.E.M. * = significant difference from control, $p < 0.05$; ** $p < 0.01$; $^{\#}$ = significant difference from Aroclor 1254, $p < 0.05$; $^{\#\#}$ $p < 0.01$. The number of litters per exposure group is given between brackets.

Discussion

The purpose of the present study was to compare the possible developmental effects caused by the PCB-metabolite 4-hydroxy-2,3,3',4',5-pentaCB (4-OH-CB107) with parent compounds, using the commercial PCB mixture Aroclor 1254. The data obtained demonstrate that prenatal exposure to 4-OH-CB107 can induce adverse developmental neurotoxic effects on its own which are similar, but also partly different from these caused by parent PCB congeners.

Maternal exposure to 4-OH-CB107 or Aroclor 1254 resulted in a significant decrease in plasma total thyroxine (TT_4) levels in both male and female offspring 4 days postpartum. The TT_4 reductions in the 5 mg/kg 4-OH-CB107 exposure group at PND 4 (approximately 34%) were less severe compared to reductions observed in fetuses at GD20 (reduction of 89%, Meerts *et al.*, submitted). This phenomenon was also observed after exposure of dams to 25 mg/kg Aroclor 1254 in

a similar experimental setup (Morse *et al.*, 1996a). The fact that Aroclor 1254 exposed offspring showed a more severe reduction in TT_4 levels at PND 4 compared to the 4-OH-CB107 exposed offspring (this study) whereas the reduction in fetal TT_4 levels was lower in Aroclor 1254 exposed compared to 4-OH-CB107 exposed fetuses (Meerts *et al.*, submitted) might be explained by kinetic differences between 4-OH-CB107 and Aroclor 1254. It is very likely that 4-OH-PCB107 will be distributed and diluted in growing neonates and eliminated faster than the parent compounds. In addition, in neonates dosed with Aroclor 1254, the production of metabolites including 4-OH-CB107 will continue, giving rise to a more continuous exposure to 4-OH-CB107 compared to 4-OH-CB107 treated offspring.

The observation that serum TSH levels in Aroclor 1254 treated offspring 4 days postpartum do not respond to the reductions in thyroxine (T_4) levels, is consistent with earlier findings (Goldey *et al.*, 1995a; Hood *et al.*, 1995; Liu *et al.*, 1995; Morse *et al.*, 1996a). Fetal TSH levels at GD20 were also not increased following Aroclor 1254 exposure (Morse *et al.*, 1996a). It is hypothesized that PCB congeners and/or their metabolites mimic thyroid hormones (Rickenbacher *et al.*, 1986; McKinney and Waller, 1994) and possibly bind to thyroid hormone receptors in the pituitary, thereby blocking TSH release. In contrast, in fetuses following maternal exposure to 5 mg 4-OH-CB107/kg body weight from gestation days 10 to 16, fetal TSH levels at GD20 were significantly increased by 124% most likely as a response to decreased T_4 levels (Meerts *et al.*, submitted). At PND 4 (this study), TSH levels in 4-OH-CB107 treated neonates were comparable to control levels in corn oil treated offspring. TSH levels were also unaffected in offspring at the age of 11 months.

The observed increase in locomotor activity in the offspring in the last 3 minutes of the trial in the open field test indicates an impaired habituation in all exposed groups. Habituation was observed in the control animals, whereas all exposed groups exhibited elevated activity levels in the last 3 min. Increased activity is a well known effect caused by PCB mixtures, ortho-substituted and coplanar congeners in rats (Lilienthal *et al.* 1990; Holene *et al.*, 1995; Jacobson and Jacobson, 1997; Schantz *et al.*, 1995; Hany *et al.* 1999b).

Also in mice, increased locomotor activity has been reported to occur in adult animals after pre- and postnatal exposure to Aroclor 1254 (Storm *et al.*, 1981), or neonatal exposure to coplanar (Eriksson *et al.*, 1991; Eriksson and Fredriksson, 1998) and *ortho*-chlorinated PCBs (Eriksson and Fredriksson, 1996). Agrawal *et al.* (1981) showed that elevated levels of locomotor activity induced by developmental exposure of mice to a high dose of 3,3',4,4'-tetrachlorobiphenyl was associated with decreased dopamine concentrations in the corpus striatum. In this study, using low to moderate doses of Aroclor 1254 and/or 4-OH-CB107, no significant changes were observed in brain dopamine concentrations in both male and female offspring exposed *in utero*. However, concentrations of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were slightly decreased in the caudate nucleus from male and female offspring exposed to 5 mg/kg 4-OH-CB107, and slightly increased in the caudate nucleus of Aroclor 1254 exposed offspring, suggesting that both 4-OH-CB107 and Aroclor 1254 are able to exert effects on dopamine metabolism or synthesis. In contrast, Morse *et al.* (1996c) showed only alterations in serotonin metabolism in rat brain following prenatal exposure to Aroclor 1254 in the same experimental setup.

The passive avoidance data indicate subtle changes in the course of latencies across the three trials following the conditioning trial. Latencies were reduced in young adult males of the low dose group treated with 4-OH-CB107, whereas in older males dose-dependent, though not significant, reductions were found 24 h after conditioning. Similar latency decreases were detected in rats prenatally exposed to 2,2',4,4'-tetraCB and, in particular, 3,3',4,4'-tetraCB (Weinand-Härer *et al.* 1997). The authors suggested that the PCB effects on thyroid hormone levels might partly explain the observed effects on neurochemical processes and behaviour. Iodine deficient rats also showed a poorer performance in the passive avoidance task, accompanied with reduced levels in T_4 and elevated TSH levels (Overstreet *et al.*, 1984). However, the fact that maternal exposure to Aroclor 1254 did not cause alterations in passive avoidance in the present study suggests that mechanisms other than reduced thyroid hormone levels during development mediate long-lasting influences on this neurobehavioural task.

The catalepsy test is a standard pharmacological test to investigate extrapyramidal side effects of neuroleptic compounds due to blocking of dopamine D2 receptors in the neostriatum. In this study, haloperidol-induced catalepsy was used to examine effects on striatal function caused by 4-OH-CB107 or Aroclor 1254. Male rats treated with 4-OH-CB107 or Aroclor 1254 showed decreases in the latencies to movement onset. This suggests alterations in the interaction between the serotonergic and dopaminergic system, since it is known that catalepsy induced by dopamine receptor antagonists can be completely antagonized by the administration of serotonin receptor agonists (Wadenberg, 1996). This is in line with the biogenic amine concentrations measured in the brain of both 4-OH-CB107 or Aroclor 1254 treated animals. Effects of developmental exposure to Aroclor 1254 on the concentrations of 5-HT and 5-HIAA are in general accordance with effects found following neonatal hypothyroidism. Savard *et al.* (1984) showed significant increases in 5-HT and 5-HIAA levels in many discrete brain nuclei in the forebrain, midbrain and hindbrain following neonatal hypothyroidism. Exposure to 4-OH-CB107 resulted in less pronounced neurochemical effects. However, the observed decreases in latencies of movement onset in the catalepsy test (this study) indicate that neurotransmitter functions may have been influenced following 4-OH-CB107 exposure aside from post mortem concentrations of neurotransmitters. The observed differences between males (late latency decreases) and females (early latency increases) may be due to differences in kinetics of haloperidol in both genders, with males showing an earlier onset of catalepsy than females, resulting in more rapid expression and decay of the response.

The precise mechanism by which Aroclor 1254 or the PCB metabolite alter concentrations of neurotransmitters is unknown. 4-OH-CB107 is known to exert anti-estrogenicity *in vitro* (Moore *et al.*, 1997; Meerts, unpublished results). High concentrations of this metabolite in the developing brain may influence CNS dopaminergic and serotonergic function, since there appear to exist interactive relationships between estrogens and DA as well as between estrogens and 5-HT (Rubinow *et al.* 1998; recent review in McEwen and Alves, 1999). Another explanation might be the recently reported extremely potent inhibition of human estrogen sulfotransferase activity (*in vitro*) by environmentally relevant hydroxylated PCBs (Kester *et al.*, 2000). The authors showed that 4-OH-CB107 was one of the strongest of the 32 tested compounds with an IC_{50} of 0.15 – 0.25 nM. This suggests that 4-OH-

CB107 might indirectly induce estrogenic activity by increasing estradiol bioavailability in target tissues.

The effects on auditory thresholds in Aroclor 1254 treated offspring are visible only in the low frequency range (500 Hz to 4 kHz). This is in line with effects observed so far concerning the influence of thyroid hormone deficiencies on auditory thresholds (Goldey *et al.* 1995a). In addition, the results shown for Aroclor 1254 treated animals are in line with results presented by Goldey *et al.* (1995b) and were related to hair cell loss in the apical part of the cochlea (Crofton *et al.* 2000a). Animals treated with 4-OH-CB107 showed no increase in BAEP thresholds, suggesting that this metabolite exerts no deleterious effects on the cochlea. However, the slight prolongation of latencies in metabolite exposed groups may indicate effects on the neural part of the auditory system. Alternatively, this may be explained by the recent observations of Crofton *et al.* (2000b), who showed in cross-fostering studies that lactational exposure to Aroclor (postnatally) is the major cause of ototoxicity

In conclusion, maternal exposure to the PCB-metabolite 4-OH-CB107 can exert adverse effects on neurotransmitter levels and brain development in rat offspring, that are both similar to and partly different from the effects observed following Aroclor 1254 exposure.

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CHAPTER 8

Summary of *in vivo* studies

In the second part of this thesis, *in vivo* studies are described which were performed to assess the consequences of prenatal exposure to hydroxylated compounds on the development of the offspring. For this purpose, the PCB metabolite 4-OH-2,3,3',4',5-pentaCB (4-OH-CB107) was selected as a model compound. 4-OH-CB107 has both thyroidogenic (see *Chapter* 5) and anti-estrogenic (Moore *et al.*, 1997) potencies *in vitro* and it is one of the major metabolites detected in human blood (Bergman *et al.*, 1994).

Before being able to determine the effects of *in utero* exposure to 4-OH-CB107 on the development of the offspring, information about the uptake and distribution of 4-OH-CB107 in pregnant rats and their fetuses was necessary. To determine these parameters, rats are usually exposed to a single dose of the compound in a radiolabelled form, and the elimination of the labelled compound is followed during a selected number of days. At the end of the study, radioactivity is determined in several organs. This study strategy is not applicable in the case of PCB-metabolites, since these compounds will normally be eliminated much faster compared to their parent compounds. In addition, the purpose of the study on the uptake and distribution of 4-OH-CB107 described in *Chapter 5* was to assess the internal exposure of fetuses to 4-OH-CB107 using the same dosing regimen as for the subsequent studies on the possible adverse effects in offspring following *in utero* exposure to 4-OH-CB107. Therefore, in the study described in *Chapter 5*, pregnant rats were exposed orally to [^{14}C]-labelled 4-OH-CB107 from gestation days 10 to 16.

The uptake of [^{14}C]-4-OH-CB107 in pregnant rats was low, i.e. 78% of the total administered dose was excreted in the faeces at gestation day 17 (GD17) and almost 94% at GD20. Despite this high faecal elimination, relatively high levels of the [^{14}C]-4-OH-CB107-derived radioactivity were determined in maternal but especially fetal tissues. Of the total dose absorbed, 51.7 ± 3.2% was present in the fetuses, indicating that the placenta forms no barrier for 4-OH-CB107. In addition, fetal levels of [^{14}C]-4-OH-CB107 in pooled livers and plasma samples were 11 times and 1.2 times higher, respectively, compared to maternal levels at GD20. As a result of 4-OH-CB107 exposure, fetal plasma TT_4 and FT_4 levels at GD20 were drastically decreased by 98% and 41%, respectively. In maternal plasma, only TT_4 levels were reduced (by 38% at GD20).

In line with the results from Morse *et al.* (1996a), fetal brain T_4 concentrations were decreased, but T_3 concentrations were unaffected. Interestingly, [^{14}C]-4-OH-CB107 derived radioactivity was bound to TTR in both maternal and fetal plasma. *In vitro* T_4-TTR competition binding studies with 4-OH-CB107 revealed a 3.3 fold higher binding potency of 4-OH-CB107 compared to the natural ligand T_4. It is suggested that this selective interaction with TTR, the major plasma thyroid hormone transport protein in rats, may explain the observed reductions in fetal thyroid hormones and the distribution of 4-OH-CB107 in the fetal compartment.

Chapters 6 and *7* describe the effects of *in utero* exposure to the PCB metabolite 4-OH-CB107 on the development of the offspring. We were interested in the comparison of the effects caused by the PCB-metabolite and effects caused by parent PCB compounds. Therefore, an additional group of rats was exposed to 25 mg/kg Aroclor 1254. Because of the drastic reductions in fetal plasma T_4 levels following exposure to 5 mg 4-OH-CB107 per kg body weight (*Chapter 5*), two metabolite dose groups were used in the studies presented in *Chapters 6* and *7*, i.e. 0.5 mg/kg and 5 mg/kg body weight. Exposure of pregnant rats to 4-OH-CB107 did not affect the development of the offspring (*Chapter 6*), as examined by recording well known developmental landmarks, such as the anogenital distance, crown-rump length, pinna detachment, and the age at the onset of hair growth, bilateral eye opening, vaginal opening (females) or preputial separation (males). The developmental effects observed following *in utero* exposure of rats to Aroclor 1254 are in line with previous findings reported in several studies. For example, Aroclor 1254 treated offspring showed a significantly earlier onset of bilateral eye opening, which has been observed by Goldey *et al.* (1995a). As discussed in *Chapter 6*, this effect is most likely caused by a direct effect of PCBs and not caused by the induced hypothyroidism, since hypothyroidism normally results in a delay of bilateral eye opening. Female offspring exposed to Aroclor 1254 showed a statistically significant increase in the anogenital distance/crown rump length ratio, which has also been observed by Goldey *et al.* (1995a) and for TCDD by Gray *et al.* (1997) and Theobald and Peterson (1997).

The most striking result presented in *Chapter 6* was the effect of 4-OH-CB107 on the estrous cycle in female offspring, which was determined from postnatal days 210 to 231. The length of the estrous cycle was dose dependently prolonged, because of an increase in the length of the diestrous stage. Aroclor 1254 exposed female offspring also had a prolongation of the estrous cycle, although less pronounced compared to 4-OH-CB107 exposed offspring, indicating that this effect is most likely induced by the metabolite. At 11 months of age, plasma estradiol levels were significantly increased in offspring exposed *in utero* to 5 mg/kg 4-OH-CB107, and unchanged in Aroclor 1254 treated offspring, again indicating that this effect might be metabolite-mediated. Possible mechanisms to explain these observations on estrous cyclicity and estradiol concentrations are discussed in *Chapter 6*.

Several subtle effects on behaviour were observed in both Aroclor 1254 and 4-OH-CB107 exposed offspring (*Chapter 7*). An increased locomotor activity compared to controls in the last 3 minutes of the trial in an open field test was observed in all treatment groups. This indicates an impaired habituation. In the passive avoidance test, very subtle differences were observed between

Aroclor 1254 exposed and 4-OH-CB107 exposed offspring. Aroclor 1254 exposed offspring showed a steady increase in latencies across the trials, whereas in all other groups a plateau was reached. In the catalepsy test, male offspring of all exposed groups showed decreases in latencies to movement onset, whereas female offspring of the exposed groups showed increases in latencies to movement onset. These differences between the sexes are difficult to explain, since many different mechanisms may play a role. Both dopamine and serotonin concentrations can affect the degree of catalepsy, and in addition, the metabolism of haloperidol may also be different in both genders resulting in e.g. earlier onset of catalepsy in males compared to females, leading to a more rapid expression and decay of the response in males.

Measurement of dopamine and serotonin concentrations in brain regions of male and female offspring revealed effects on both dopaminergic and serotonergic systems in Aroclor 1254 exposed offspring. Serotonin (5-hydroxytryptamine, 5-HT) and its metabolite, 5-hydroxy-indoleacetic acid (5-HIAA) were significantly increased in the frontal cortex of male offspring exposed *in utero* to Aroclor 1254 compared to the levels in control animals. In addition, the ratio between 3,4-dihydroxyphenylacetic acid (the dopamine metabolite DOPAC) and dopamine (DA) in the caudate nucleus of male offspring of the Aroclor 1254 exposure group was significantly increased. The same trends, although not significant, could be observed in female offspring. Strikingly, treatment of rats to 4-OH-CB107/kg per day from GD10 to 16 resulted in the offspring in changes in the dopaminergic system only. The concentration of the dopamine metabolites DOPAC and homovanillic acid (HVA) were decreased in the nucleus accumbens and caudate nucleus, respectively, in male offspring of the 5 mg/kg 4-OH-CB107 treatment group. Increases in 5-HT and 5-HIAA have also been shown in brain regions of rats following neonatal hypothyroidism. However, the fact that these increases were not observed in 4-OH-CB107 treated rats favours the explanation that in case of Aroclor 1254 treated offspring a direct effect of parent PCB congeners on serotonin metabolism may lead to changes in the concentrations of both 5-HT and 5-HIAA, instead of the indirect effect caused by the Aroclor induced hypothyroidism.

Effects on auditory thresholds (*Chapter 7*) were mainly observed for Aroclor 1254 treated offspring, and are in line with the results described by Goldey *et al.* (1995b). In 4-OH-CB107 treated offspring, only a slight prolongation of the latencies was observed, but no effects could be seen on the brain stem auditory thresholds. This is in line with the recent publication of Goldey *et al.* (2000b) who were able to show that postnatal hypothyroidism caused by exposure to Aroclor 1254 is the major cause of ototoxicity (Goldey *et al.*, 2000b).

In conclusion, prenatal exposure to the PCB metabolite 4-OH-CB107 can result in endocrine disrupting effects and effects on neurobehaviour, which are quite similar but in some ways different from the effects observed following Aroclor 1254 exposure. Metabolite-induced effects are (i) decreases in fetal and neonatal thyroid hormone levels, (ii) increases in the length of the estrous cycle in female offspring, (iii) increased estradiol concentrations in female offspring in the pro-estrous stage at the age of 11 months, (iv) alterations in brain dopamine metabolism and (v) slight prolongations of latencies in brain stem auditory evoked potentials. Table 8.1 summarizes the differences in effects observed for Aroclor 1254 and 4-OH-CB107 presented in this thesis.

Table 8.1. Comparison of the effects observed following *in utero* exposure to 4-OH-CB107 or Aroclor 1254.

Observation	**4-OH-CB107**	**Aroclor 1254**
AGD/CRL ratio (PND4)	no effect	↑ in ♀
Day of eye opening	no effect	accelerated
Estrous cycle length in female offspring (PND210-231)	↑↑ (prolonged)	↑ (prolonged)
Plasma E_2 in female offspring (11 months old)	↑↑	no effect
Fetal TT_4 (GD20)	↓↓↓	↓↓ [a]
Fetal FT_4 (GD20)	↓↓	↓↓↓ [a]
Fetal TSH (GD20)	↑↑	no effect [a]
Maternal TT_4 (GD20)	↓	↓↓ [a]
Maternal FT_4 (GD20)	No effect	↓↓ [a]
Maternal TT_3 (GD20)	No effect	↓ [a]
Neonatal TT_4 (PND4)	↓↓	↓↓↓
Neonatal FT_4 (PND4)	No effect	No effect
Neonatal TT_3 (PND4)	No effect	↓ (only significant in ♂)
Locomotor activity at PND130	Impaired habituation in ♂	Impaired habituation in ♂
Catalepsy	Reduced latencies (grid, ♂); Increased latencies (grid, ♀)	Reduced latencies (grid, ♂). ♀: no significant effects.
BAEP: auditory thresholds	No effects	Threshold elevated at low frequencies (0.5, 1 and 2 kHz)
BAEP: peak latencies	Prolongation of peak II latencies[b]	No effect
Brain biogenic amines	Effects on dopaminergic system (DOPAC and HVA increased in CN)	Effects on dopaminergic (DOPAC/DA increased in CN) and serotonergic system (5-HIAA and 5-HT increased in FC)

[a] Presented in Morse *et al.*, 1996a.

[b] Effect was not statistically significant.

CHAPTER 9

General discussion

The research presented in the first part of this thesis (*Chapters 2, 3 and 4)* describes a number of *in vitro* studies on the possible endocrine active potencies of polybrominated diphenyl ethers and related brominated aromatic hydrocarbons, fairly new compounds of which some have already been identified in human blood. The *in vivo* studies described in the second part of this thesis were performed to assess the possible long-term adverse effects of exposure to endocrine active compounds in rats. For this purpose, a model compound (4-hydroxy-2,3,3',4',5-pentachlorobiphenyl, 4-OH-CB107) with *in vitro* thyroidogenic and anti-estrogenic potencies was chosen, and the studies were focused on fetal and neonatal development, since the developing fetus is extremely vulnerable to changes in the endocrine system (*Chapters 5, 6, 7 and 8).*

In vitro endocrine active potencies of polybrominated diphenyl ethers and related compounds

The *in vitro* T_4-TTR competition binding studies revealed that hydroxylated brominated flame retardants of several different classes were able to bind to human TTR. Compounds with the highest binding potency were TBBPA and PBP, both compounds having bromine substituents next to the hydroxyl group. An important conclusion of the results obtained on brominated diphenyl ethers was that metabolic conversion to most likely hydroxylated PBDEs is a prerequisite for interactions with TTR. This conclusion is supported by the fact that the higher brominated diphenyl ethers, which will probably not be metabolized because of the steric hindrance of the extensive bromine substituents, were not capable to interact with TTR before or after metabolic conversion. In addition, PBDEs as parent compounds did not show any competition with T_4 before metabolic activation, whereas after metabolic activation all di- to pentabrominated diphenyl ethers tested showed T_4-TTR competition binding potencies. Unfortunately, hydroxylated PBDEs of these congeners were not available, so it was not possible to determine the structure affinity relationships for hydroxylated PBDEs.

The structure affinity relationships that were deduced from the brominated (bis)phenols in *Chapter 2* are in agreement with earlier reports from Den Besten *et al.* (1991), van den Berg (1990), Brouwer *et al.* (1990), Cheek *et al.* (1999), Lans *et al.* (1993) and Rickenbacher *et al.* (1986). In addition, X-ray crystallography studies revealed a new mode of binding of the single ring compounds pentabromophenol (PBP) and 2,4,6-tribromophenol (TBP) to TTR (summarized in *Chapter 4*; published by Ghosh *et al.*, 2000). In these studies it was observed that PBP and TBP bound to TTR in a reversed mode, e.g. a bromine atom positioned in the centre of the TTR binding channel instead of the hydroxy-group, which is the more common mode of binding. These data indicate that the hydroxyl group is not always a prerequisite for binding of a compound to TTR. Consequently, it can

thyroid hormone levels, can influence development both *in utero* and postnatally, through transplacental and lactational passage. The lactational transfer of PCB metabolites will be much lower or absent, resulting in only *in utero* exposure in case of pure PCB metabolites. Goldey *et al.* (2000b) were able to show that ototoxicity is especially caused by postnatal and not prenatal exposure of rats to Aroclor 1254.

Implications of exposure to especially thyroidogenic compounds for human health

An important question in toxicological studies performed in laboratory animals is, if the effects that are observed in rats will also be relevant for the human situation. In rats, transthyretin is the major thyroid hormone transport protein in the blood. In humans however, thyroid hormone binding globulin (TBG) is the major thyroid hormone binding protein and interaction studies with hydroxylated PCBs and TBG revealed that the OH-PCBs that were able to interact with TTR, showed no interaction at all with TBG (Lans *et al.*, 1994). This would imply that binding of a compound to TTR will very probably not have consequences for human health. However, TTR is known to be the major thyroid hormone binding protein in the cerebrospinal fluid (CSF) and it is very probably the main transporter of T_4 in the placenta. Given the fact that most hydroxylated PCBs that have been detected in human blood have T_4-TTR competition binding potencies and their structures fulfil the structural requirements for binding of a compound to TTR (see also *Chapter 1*), it can not be excluded that TTR binding in the cerebrospinal fluid and placenta may facilitate the uptake of toxic PCB metabolites in the brain and fetus and may also have adverse effects on the kinetics of T_4. Indeed, the human placenta does not seem to be a barrier for hydroxylated PCB-metabolites. Studies in human cord blood and maternal blood revealed that the ratio of hydroxylated compounds in cord blood (representing fetal blood) compared to maternal blood is approximately 1, indicating that OH-PCBs can cross the placenta very easily. The ratio of parent PCBs in cord blood compared to maternal blood is below 0.5, thus parent PCBs are transported to the human fetus in a much smaller amount than their metabolites (Bergman *et al.*, 1999b).

In the 1980s, human studies have also confirmed that PCBs could pass the placenta and that higher cord serum PCB levels were associated with lowered birth weights (Fein *et al.*, 1984). In addition, children with higher *in utero* PCB exposure showed delayed central nervous system functioning (Jacobson *et al.*, 1990) and reductions in cognitive function at the age of 4 years (Jacobson and Jacobson, 1993). A study performed in the Netherlands showed a statistically significant negative correlation between human milk dioxin and PCB-levels and plasma T_4 and T_3 levels (Koopman-Esseboom *et al.*, 1994). It is not clear if the effects of PCBs on thyroid hormone levels and metabolism may have had consequences for brain development in humans, or that hydroxy-PCBs themselves may have affected human brain development directly.

It is too early to predict the possible consequences of the observed effects on estrous cyclicity in female rat offspring for the human situation. Despite the prolonged diestrous stage in female rats, no adverse effects could be seen on the reproductive capacity of these females (e.g. the number of matings attempted) or on their fetuses (no effects on the number of resorptions or

implantation sites, the number of dead or life fetuses, total litter weight, mean fetal body weight or sexe ratio). However, in our study we did not investigate the development of the fetuses. More detailed studies on the development of the F_2-offspring will be necessary to determine if there are any consequences on reproduction. In addition, studies on the sexual development of the brain in female rats exposed *in utero* will help to discriminate between effects caused by 4-OH-CB107 directly, or effects that are related to the changes in steroid or thyroid hormones. Preliminary research on the number of spinal nuclei of the Bulbocavernosus (SNB), a sexually dimorphic nucleus in the ventral horn of the fifth and sixth lumbal segments of the rat spinal cord, revealed that the number of SNB cells in females exposed to 5 mg/kg 4-OH-CB107 was significantly higher compared to control group. In males, exposure to 4-OH-CB107 or Aroclor 1254 both resulted in a significant higher number of SNB cells compared to corn oil treated rats (van der Beek, unpublished results). The SNB contains motor neurons whose axons innervate the bulbocavernosus muscle, which is attached to the base of the male penis. The SNB is almost absent in female rats. Injection of female rats with testosterone on PND2 results in the development of the SNB (Breedlove *et al.*, 1982). The higher number of SNB cells in females exposed to 4-OH-CB107 suggests that 4-OH-CB107 may increase endogenous androgen production, since androgen and not estrogen concentrations regulate the number of SNB cells (Breedlove, 1997). However, in this case one would also expect to see effects on the weights of the male accessory organs, and this was not observed (*Chapter 6*). To clarify the mechanism and the onset of the observed effects regarding e.g. the sexual development of the brain (number of SNB cells) and the irregular cyclicity of the female offspring, further studies should be focused on the sexual development and steroid hormone levels in exposed offspring during different stages of life (e.g. neonatal, puberty, early adult and adult stages). Although these mechanisms are not clarified yet, it can be stated that in case PCB-metabolites are also able to induce reproductive senescence in humans at an earlier stage of life, this may have serious effects on human reproduction, especially in a time period when the average age of females having their first child is increasing.

Human individuals as well as wildlife species are unintentionally exposed to numerous chemicals of anthropogenic origin. Unfortunately, most of these man-made compounds have been neither tested nor evaluated for their hazard potential. In 1990, the European Union published the so-called EINECS list listing 100106 "existing substances" that were on the European market during 1971 to 1981 (European Inventory of Existing Chemical Substances). For only about 5000 of these chemicals the data needed to evaluate many of their potential effects on human health and/or the environment exist (Vallack *et al.*, 1998). In addition, only for a few hundred of these chemicals there is sufficient knowledge to perform a full hazard assessment. Although data on most of the toxicity endpoints are available, only very limited data is available for chronic endpoints such as effects of a compound on the endocrine system. The results presented in this thesis show that thyroidogenic compounds, including hydroxylated PCBs, may be able to exert adverse effects on brain development and reproduction. Especially on the reproductive system, the hydroxylated PCB showed more effects compared to the parent compounds. Humans are exposed to both parent PCB congeners and their metabolites. Exposure to parent PCB congeners will lead to a continuous exposure to metabolites, since these will be formed via metabolism *in vivo*.

The concentration of the metabolite determined in fetal plasma of rats exposed to 5 mg/kg 4-OH-CB107 at gestation day 20 was 12.7 μg/g wet weight. Considering the fact that the average number of fetuses per dam was 13 in this study, this would mean that each fetus was exposed to approximately 0.98 μg 4-OH-CB107 per g wet weight. A recent study of Sandau (2000) revealed that the concentration of total PCB-metabolites measured in umbilical cord plasma from Canadian women was in the range of 103 – 1750 pg/g wet weight. Thus, OH-PCB concentrations in the umbilical cord of humans are still at least 560 times lower compared to the levels determined in rats exposed *in utero* to 5 mg/kg 4-OH-CB107 (see *Chapter 5*). However, in rats exposed to 0.5 mg/kg 4-OH-CB107 effects on behaviour and estrous cyclicity were also visible, whereas the difference between plasma levels of these rats with the human situation at the moment is only one order of magnitude.

Overall, it can be concluded that continuing exposure to compounds such as PBDEs, PCBs and their metabolites, as well as halogenated phenols, which show an endocrine disrupting potency (e.g. thyroidogenic and estrogenic) may pose a threat on the development of human infants. Although PBDEs have a lower impact on e.g. thyroid hormone levels in rodents (Zhou *et al.*, 2001) compared to coplanar PCBs (Seo *et al.*, 1995), they may add to the effects caused by 'historic' contaminants like PCBs. Preliminary data published by Hallgren and Darnerud (1998) in fact show some evidence that co-administration of PBDEs and PCBs (Aroclor 1254) or chlorinated paraffins has an additive effect on the reduction of T_4 levels. Therefore, future research aimed at finding possible associations between exposure to organohalogen substances and the effects on human development should include phenolic metabolites as well as their parent congeners. In case of endocrine active compounds, there is some concern that pregnant women may be at risk for possible hormone disruption, since developing fetuses and infants are especially responsive to small changes in e.g. thyroid hormone levels. An additional risk factor that has to be kept in mind in case of thyroid hormone disrupting compounds are women with iodine deficiency. About twelve percent of the general population and about 15 percent of women of childbearing age in the United States are iodine deficient (Hollowell *et al.*, 1998). It is thus recommended that screening of compounds for their possible endocrine active potencies is included in the testing protocols preferably at an earlier stage of product development in order to prevent the release of these substances onto the market.

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Samenvatting

Het onderzoek dat in het eerste deel van dit proefschrift is beschreven (*Hoofdstukken 2,3 en 4*) betreft enkele *in vitro* studies, die zijn uitgevoerd om mogelijke endocriene werking van polygebromeerde difenyl ethers en gebromeerde aromatische koolwaterstoffen te onderzoeken. Deze stoffen zijn relatief recent in het milieu aangetoonde verbindingen, waarvan enkele inmiddels in humaan bloed zijn aangetoond. De *in vivo* studies, beschreven in het tweede deel van dit proefschrift, zijn uitgevoerd om de mogelijke lange termijn effecten te onderzoeken van blootstelling van ratten aan endocrien actieve verbindingen. Voor dit doel is gebruik gemaakt van een modelverbinding (4-hydroxy-2,3,3',4',5-pentachloorbifenyl, 4-OH-CB107) waarvan bekend is dat deze *in vitro* schildklierhormoon-achtige en anti-oestrogene werking vertoont. De *in vivo* studies waren vooral gericht op de foetale en neonatale ontwikkeling, omdat de ontwikkelende foetus extreem gevoelig is voor veranderingen in het hormonale systeem (*Hoofdstukken 5, 6, 7 en 8*).

In vitro endocriene activiteit van polygebromeerde difenyl ethers en hieraan gerelateerde verbindingen

De *in vitro* T_4-TTR verdringingsstudies toonden aan dat gehydroxyleerde gebromeerde vlamvertragers uit verschillende klassen kunnen binden aan humaan TTR. Verbindingen met de hoogste verdringingsactiviteit zijn TBBPA en PBP, beide stoffen bevatten broomsubstituenten direct naast de hydroxy-groep. Een belangrijke conclusie van de resultaten die verkregen zijn met de gebromeerde difenyl ethers in de T_4-TTR verdringingsstudies is dat metabole omzetting tot hoogstwaarschijnlijk gehydroxyleerde PBDEs een vereiste is voor interactie van PBDEs met TTR. Deze conclusie wordt ondersteund door het feit dat de hoger gebromeerde difenyl ethers die waarschijnlijk niet gemetaboliseerd worden als gevolg van de sterische hindering door de grote broomsubstituenten, geen interactie vertonen met TTR voor of na metabole omzetting. Tevens vertonen PBDEs als uitgangsstof geen competitie met T_4 vóór metabole activatie, terwijl na metabole activatie alle geteste di- tot pentagebromeerde difenyl ethers enige T_4-TTR verdringing bezitten. Helaas waren er op het moment van het onderzoek geen gehydroxyleerde PBDEs van deze congeneren beschikbaar, zodat het niet mogelijk was een struktuur-affiniteitsrelatie van gehydroxyleerde PBDEs af te leiden.

De struktuur-affiniteitsrelaties die kunnen worden afgeleid uit de studies met gebromeerde (bis)fenolen in *Hoofdstuk 2* zijn in overeenstemming met eerdere bevindingen van Den Besten *et al.* (1991), van den Berg (1990), Brouwer *et al.* (1990), Cheek *et al.* (1999), Lans *et al.* (1993) en Rickenbacher *et al.* (1986).

Met behulp van röntgendiffractie kristallografie werden tevens nieuwe bindingskarakteristieken ontdekt bij de binding van de enkele-ring verbindingen pentabroomfenol (PBP) en 2,4,6-tribroomfenol (TBP) aan TTR (samengevat in *Hoofdstuk 4*; gepubliceerd in Ghosh *et al.*, 2000). Uit deze studies bleek dat PBP en TBP aan TTR binden in een zogenaamde 'omgekeerde' volgorde, met een broomatoom in het centrum van het bindingskanaal van het TTR eiwit, in plaats van de hydroxy-groep die zich normaal gesproken in deze positie bevindt. Deze resultaten tonen aan dat een hydroxy-groep niet altijd een vereiste is voor de binding van een stof aan TTR. Derhalve kan worden geconcludeerd dat meerdere verbindingen met T_4 een interactie kunnen aangaan voor TTR binding dan tot nu toe werd aangenomen.

De resultaten met de gebromeerde difenyl ethers en de gebromeerde bisphenol A derivaten in de ER-CALUX studies toonden aan dat zeven van de 17 onderzochte PBDEs een zodanige oestrogene activiteit bezitten dat EC_{50}-waarden bepaald konden worden. De extracten van PBDEs na microsomale incubatie met de verschillend geïnduceerde microsomen zijn ook onderzocht volgens de methode die beschreven is in *Hoofdstuk 2*. Echter, in deze microsomale extracten kon geen enkele oestrogene werking gemeten worden (de resultaten zijn niet getoond). De mogelijkheid bestaat dat de concentratie van de metabolieten in deze extracten te laag was om een meetbaar oestrogeen effect te verkrijgen. Dit is het gevolg van de grote verdunning die nodig is in de ER-CALUX studies (1000 maal) en het feit dat de oestrogene werking van verbindingen zeer laag is ten opzichte van bijvoorbeeld de schildklierhormoon-achtige werking,. De oestrogene werking van de PBDEs met oestrogene activiteit is een orde van grootte lager vergeleken met de bekende oestrogene stof bisfenol A. De oestrogene werking van de gesynthetiseerde gehydroxyleerde PBDEs T_2-OH-BDE en T_3-OH-BDE is in dezelfde orde van grootte als bisfenol A. Zoals reeds bediscussieerd in *Hoofdstuk 4* bezitten de drie anti-oestrogene PBDEs (BDE-153, -166 en -190) ook dioxine-achtige werking. Een andere interessante bevinding is, dat de oestrogene werking van T_2-OH-BDE afhankelijk is van de oestrogeen receptor. De oestrogene werking van deze OH-BDE was in de 293-ERα cellijn hoger dan in de 293-ERβs-luc cellijn.

Nadelige lange termijn effecten na *in vivo in utero* blootstelling aan een gehydroxyleerde PCB-metaboliet met schildklierhormoonachtige en anti-oestrogene werking

Het onderzoek dat is beschreven in de *Hoofdstukken 5, 6 en 7* is uitgevoerd om een schatting te kunnen maken van de mogelijke nadelige gevolgen van *in utero* blootstelling aan endocrien actieve verbindingen in ratten. Er werd verondersteld dat binding van stoffen aan TTR zou kunnen leiden tot een vergemakkelijkt transport van de stof van de moeder naar de foetus. Er was tot op dat moment slechts een beperkt aantal studies uitgevoerd waarin deze veronderstelling is onderzocht, en bijna geen enkele studie was gericht op de mogelijke lange termijn effecten van *in utero* blootstelling aan een stof die T_4 van het TTR kan verdringen.

Om deze vraag te beantwoorden zijn twee *in vivo* studies uitgevoerd, waarin drachtige ratten werden blootgesteld aan een PCB-metaboliet (4-OH-2,3,3',4',5-pentaCB, 4-OH-CB107) gedurende dag 10 tot en met 16 van de dracht. In de eerste *in vivo* studie is de maternale en foetale

verdeling van de PCB-metaboliet onderzocht. Uit deze studie bleek, dat er in het foetale deel hoge concentraties van 4-OH-CB107 aangetoond konden worden. Tevens waren maternale, maar vooral foetale TT_4-niveaus drastisch verlaagd. Deze resultaten, samen met de detectie van de stof gebonden aan TTR in maternaal en foetaal plasma, verifieerden de hypothese dat binding van een stof aan TTR kan leiden tot een vergemakkelijkt transport van deze stof van de moeder naar de foetus, en derhalve kan resulteren in verlaagde plasma T_4-niveaus in moeder en foetus (*Hoofdstuk 5*). Er kan echter niet worden geconcludeerd dat alle verbindingen die *in vitro* aan TTR kunnen binden dezelfde effecten zullen laten zien als 4-OH-CB107. De mogelijkheid om *in vivo* effecten te veroorzaken is sterk afhankelijk van de kinetiek van een stof. Tetrabroombisfenol A (TBBPA), een stof met een zeer hoge *in vitro* T_4-TTR verdringingsactiviteit, gaf *in vivo* geen veranderingen in foetale thyroxineniveaus na blootstelling van drachtige ratten gedurende dag 10 tot en met dag 16 van de dracht. Waarschijnlijk wordt dit veroorzaakt door de lage opname van TBBPA in ratten via orale toediening (Meerts *et al.*, 1999).

In de tweede *in vivo* studie zijn de lange termijn effecten van *in utero* blootstelling aan 4-OH-CB107 onderzocht en vergeleken met de effecten die veroorzaakt worden door een commercieel mengsel van PCBs, Aroclor 1254 (*Hoofdstukken 6 en 7*). De lange termijn effecten veroorzaakt door 4-OH-CB107 op de oestrus cyclus, neurotransmitter concentraties in de hersenen, en gedrag in de nakomelingen waren wel degelijk anders dan de effecten veroorzaakt door Aroclor 1254 (samengevat in *Hoofdstuk 8*). Het meest opzienbarende effect van de PCB-metaboliet was het effect op de oestrus cyclus, waargenomen in ratten van 7-8 maanden oud. Omdat de effecten op de oestruscyclus duidelijker waren in 4-OH-CB107 behandelde nakomelingen in vergelijking met Aroclor 1254 behandelde nakomelingen, kan worden geconcludeerd dat dit effect voornamelijk wordt veroorzaakt door prenatale blootstelling van ratten aan 4-OH-CB107. De seksuele cyclus van een jonge rat duurt normaal gesproken 4-5 dagen, en wordt onregelmatig naarmate de rat ouder wordt, vanaf ongeveer 200 dagen en ouder. Neonatale blootstelling aan oestrogene stoffen (bijvoorbeeld oestradiol benzoaat vanaf de geboorte gedurende 10 of meer opeenvolgende dagen) leidt direct na de vaginale opening tot het continue di-oestrus syndroom (Aihara en Hayashi, 1989). Het is niet bekend of de oestruscyclus al verstoord was ten tijde van de vaginale opening in de nakomelingen die zijn blootgesteld aan 4-OH-CB107, omdat de oestrus cyclus alleen in de volwassen vrouwtjesrat is onderzocht. De hogere oestradiol (E_2)-concentratie in plasma van nakomelingen (vrouwtjes) van de groep blootgesteld aan 5 mg/kg 4-OH-CB107 komt overeen met hogere E_2-niveaus die gevonden worden in volwassen ratten in het continue di-oestrus stadium. Voordat de cyclus onregelmatig wordt laat een volwassen vrouwtjesrat een verlaagde LH-reactie zien als gevolg van het positieve terugkoppelingseffect van E_2. Men denkt dat hogere E_2-concentraties in volwassen ratten tijdens opeenvolgende oestruscycli uiteindelijk de neuro-endocriene reactie van het terugkoppelingseffect van E_2 op LH secretie vermindert (Lu *et al.*, 1994). Tevens bestaat het vermoeden dat dit proces versneld kan worden door een grote concentratie oestrogeen vlak voor of na de geboorte (Hayashi en Aihara, 1989). Concluderend uit deze gegevens is het mogelijk dat blootstelling van vrouwtjesratten aan 4-OH-CB107 kan leiden tot een vervroegde reproductieve veroudering in vergelijking met ratten die zijn blootgesteld aan maïsolie.

Uit de resultaten van de *in vivo* studie beschreven in *Hoofdstukken 6 en 7* kan worden geconcludeerd dat blootstelling van ratten aan een gehydroxyleerde PCB-metaboliet kan leiden tot nadelige gedragseffecten. Er kan echter niet worden geconcludeerd dat negatieve effecten op de hersenontwikkeling het gevolg zijn van de hypothyroïde staat tijdens de ontwikkeling door blootstelling aan 4-OH-CB107, of het gevolg van directe interacties van 4-OH-CB107 op (het metabolisme van) de neurotransmitters in de hersenen. De concentraties 4-OH-CB107 in foetaal plasma op dag 20 van de dracht zijn hoger in ratten die *in utero* zijn blootgesteld aan 5 mg/kg 4-OH-CB107 (deze studie) dan de *in vivo* gevormde 4-OH-CB107 concentraties in ratten na blootstelling aan Aroclor 1254 (Morse *et al.*, 1996a). De effecten van Aroclor 1254 op de hersenontwikkeling en het gehoor zijn duidelijker dan de effecten veroorzaakt door 4-OH-CB107. Dit kan worden verklaard door het feit dat de blootstelling van stoffen die schildklierhormoon-niveaus kunnen verlagen zoals PCBs en dioxines via transplacentale overgang en via de moedermelk kan plaatsvinden en derhalve de ontwikkeling *in utero* en postnataal kan beïnvloeden. Dit laatste zal in geval van PCB-metabolieten lager of zelfs afwezig zijn, resulterend in enkel *in utero* blootstelling bij zuivere PCB-metabolieten. Goldey *et al.* (2000b) toonden aan dat gehoortoxiciteit specifiek veroorzaakt wordt door postnatale en niet prenatale blootstelling van ratten aan Aroclor 1254.

Gevolgen van blootstelling aan vooral schildklierhormoon-achtige verbindingen voor de gezondheid van de mens

Een belangrijke vraag bij toxicologische studies die zijn uitgevoerd op laboratorium dieren is of de effecten die zijn waargenomen in ratten ook relevant zullen zijn voor de humane situatie. In ratten is transthyretine het belangrijkste eiwit voor het transport van schildklierhormonen in het bloed. In de mens is dit echter thyroxine bindend globuline (TBG), en interactie studies met gehydroxyleerde PCBs en TBG toonden aan dat de OH-PCBs die interactie vertoonden met TTR, geen enkele interactie vertoonden met TBG (Lans *et al.*, 1994). Dit zou betekenen dat de binding van een stof aan TTR waarschijnlijk geen gevolgen zou hebben voor de gezondheid van de mens. Echter, TTR is het belangrijkste schildklierhormoon bindend eiwit in de cerebrospinale vloeistof (CSF) en het is hoogst waarschijnlijk het belangrijkste transport van T_4 in de placenta. Gezien het feit dat de meeste gehydroxyleerde PCBs die in het (humane) bloed worden aangetoond T_4-TTR verdringing laten zien en hun structuren voldoen aan de structurele voorwaarden van een stof voor binding aan TTR (zie ook *Hoofdstuk 2*), kan het niet worden uitgesloten dat TTR binding in de cerebrospinale vloeistof en de placenta de opname van toxische PCB metabolieten in de hersenen en de foetus kan vergemakkelijken, en tevens een effect kan hebben op de kinetiek van T_4. De humane placenta blijkt inderdaad geen barrière te zijn voor gehydroxyleerde PCB-metabolieten. Onderzoek naar de concentraties van gehydroxyleerde verbindingen in humaan navelstrengbloed (representatief voor foetaal bloed) en maternaal bloed toonde aan dat de ratio van gehydroxyleerde verbindingen in navelstrengbloed in vergelijking met maternaal bloed ongeveer 1 is. Dit betekent dat OH-PCBs de placenta erg makkelijk kunnen passeren. De ratio tussen PCBs in navelstrengbloed en maternaal bloed is lager dan 0.5, waaruit blijkt dat PCBs zelf in een veel lagere hoeveelheid worden getransporteerd van de moeder naar de foetus dan de metabolieten (Bergman *et al.*, 1999b).

In de tachtiger jaren hebben humane studies ook bevestigd dat PCBs de placenta kunnen passeren. Tevens werden hogere PCB concentraties in navelstreng serum geassocieerd met lagere geboortegewichten (Fein *et al.*, 1984). Kinderen met een hogere *in utero* blootstelling aan PCB vertoonden vertraagde functies van het centrale zenuwstelsel (Jacobson *et al.*, 1990) en verlagingen in de cognitieve functie op de leeftijd van 4 jaar (Jacobson en Jacobson, 1993). Een in Nederland uitgevoerde studie toonde een statistisch significante negatieve correlatie aan tussen dioxine- en PCB-concentraties in humane melk en plasma T_4 en T_3 niveaus (Koopman-Esseboom *et al.*, 1994). Het is niet duidelijk of de effecten van PCBs op de schildklierhormoon-niveaus en metabolisme hebben geleid tot de gevolgen voor de humane hersenontwikkeling, of dat de hydroxy-PCBs zelf de humane hersenontwikkeling direct hebben beïnvloed.

Het is nog te vroeg om te voorspellen wat de mogelijke gevolgen van de waargenomen effecten op de oestrus cyclus in de vrouwelijke nakomelingen van de ratten zullen zijn voor de humane situatie. Ondanks het verlengde di-oestrus stadium in vrouwtjes ratten zijn geen nadelige effecten waargenomen wat betreft de reproductiecapaciteit van deze dieren (zoals bijvoorbeeld het aantal paringen dat noodzakelijk was) of op hun foetussen (geen effect op het aantal resorpties of implantatieplaatsen, het aantal dode of levende foetussen, totale nestgewicht, gemiddelde foetale gewicht of seksratio). In deze studie is echter niet gekeken naar de ontwikkeling van de foetussen. Om te bepalen of er enige gevolgen zijn voor de voortplanting is het noodzakelijk om meer gedetailleerde studies uit te voeren waarin de ontwikkeling van de F_2-nakomelingen nader wordt onderzocht. Tevens zullen studies gericht op de seksuele ontwikkeling van de hersenen van *in utero* blootgestelde vrouwtjesratten helpen om een onderscheid te kunnen maken tussen de effecten die rechtstreeks veroorzaakt zijn door 4-OH-CB107 en effecten die het gevolg zijn van de veranderingen in steroïd- of schildklierhormonen.

Voorlopige resultaten van een onderzoek naar het aantal spinale kernen van de Bulbocavernosus (SNB), een seksueel dimorfe kern die zich in het ventrale deel tussen het vijfde en zesde segment van de ruggengraat van de rat bevindt, toonden aan dat het aantal SNB cellen in vrouwtjes blootgesteld aan 5 mg/kg 4-OH-CB107 significant verhoogd was ten opzichte van de controle groep. In mannetjes resulteerde de blootstelling aan 4-OH-CB107 of Aroclor 1254 beide in een significant verhoogd aantal SNB cellen ten opzichte van controle dieren (van der Beek, persoonlijke communicatie). De SNB bevat motor neuronen waarvan de axonen in de spier van de bulbocavernosus eindigen. Deze spier is gehecht aan de basis van de mannelijke penis. De SNB is bijna geheel afwezig in vrouwtjesratten. Injectie van vrouwtjesratten met testosteron op de 2e dag na de geboorte (PND 2) resulteert in de ontwikkeling van de SNB (Breedlove *et al.*, 1982). Het grotere aantal SNB cellen in vrouwtjes blootgesteld aan 4-OH-CB107 suggereert dat 4-OH-CB107 mogelijk in staat is de endogene androgeen productie te verhogen, daar androgeen concentraties (en niet oestrogeen concentraties) het aantal SNB cellen reguleert (Breedlove, 1997). Als dit het geval is zou men echter ook effecten verwachten op de gewichten van de mannelijke geslachtsorganen, en dit kon niet worden aangetoond (*Hoofdstuk 6*). Om dit mechanisme op te helderen en tevens de start van de waargenomen effecten betreffende de seksuele ontwikkeling van het brein (aantal SNB cellen) en de onregelmatige oestrus cyclus van de vrouwtjes nakomelingen te onderzoeken, zouden vervolgstudies gericht moeten worden op de seksuele ontwikkeling waarbij steroïdhormoon niveaus in blootgestelde

nakomelingen van verschillende leeftijd worden onderzocht (bijvoorbeeld neonataal, of tijdens de puberteit, het vroege volwassen en volwassen stadium). Hoewel deze mechanismen nog niet zijn opgehelderd kan worden geconcludeerd dat indien PCB-metabolieten in staat zijn vervroegde reproductieve veroudering ook te kunnen induceren bij mensen, dit ernstige gevolgen kan hebben voor de voortplanting van de mens, vooral nu de gemiddelde leeftijd waarop een vrouw haar eerste kind krijgt steeds hoger wordt.

Mensen, maar ook in het wild levende dieren worden onbedoeld blootgesteld aan veel verschillende chemicaliën die gemaakt zijn door de mens. Tot dusver is het grootste deel van deze verbindingen niet getest of geëvalueerd op hun mogelijke gevaren. In 1990 heeft de Europese Unie een lijst gepubliceerd (de EINECS lijst, de Europese Inventaris voor bestaande Chemische Stoffen) waarin 100106 bestaande stoffen genoemd worden die op de Europese markt waren gedurende 1971 tot 1981. Voor slechts ongeveer 5000 van deze stoffen zijn resultaten beschikbaar die nodig zijn voor de evaluatie van de mogelijke effecten op de humane gezondheid en/of het milieu (Vallack *et al.*, 1998). Van slechts enkele honderden van deze chemicaliën is voldoende bekend om een volledige risicoschatting te maken. Hoewel de resultaten van de meeste toxicologische eindpunten beschikbaar zijn, is er slechts weinig bekend van de chronische eindpunten zoals de effecten van een stof op het endocriene systeem. De resultaten die beschreven zijn in dit proefschrift tonen aan dat schildklierhormoon-achtige verbindingen, waaronder gehydroxyleerde PCBs, een mogelijk nadelige invloed kunnen hebben op de hersenontwikkeling en de voortplanting. Vooral op het gebied van de voortplanting gaven de gehydroxyleerde PCBs meer effecten in vergelijking met hun uitgangsstoffen. Mensen worden naast deze uitgangsstoffen (de PCB congeneren) ook blootgesteld aan hun metabolieten. Blootstelling aan de uitgangsstof kan tevens leiden tot een continue blootstelling aan metabolieten, omdat deze via metabolisme *in vivo* gevormd worden.

De concentratie van de metaboliet in het plasma van foetale ratten, die zijn blootgesteld aan 5 mg/kg 4-OH-CB107, op dag 20 van de dracht was 12.7 µg/g nat gewicht. Gezien het feit dat het gemiddelde aantal foetussen per rat 13 was in deze studie, zou dit betekenen dat elke foetus blootgesteld is aan ongeveer 0.98 µg 4-OH-CB107 per g nat gewicht. Een recente studie van Sandau (2001) toonde aan dat de totale PCB-metaboliet concentraties, gemeten in navelstreng plasma van Canadese vrouwen, varieert van 103 -1750 pg/g nat gewicht. OH-PCB concentraties in humaan navelstreng bloed zijn dus nog steeds ten minste 560 keer lager dan de concentraties die gevonden zijn in de ratten na *in utero* blootstelling aan 5 mg/kg 4-OH-CB107 (zie *Hoofdstuk 5*). Echter, ook in de ratten blootgesteld aan 0.5 mg/kg 4-OH-CB107 waren effecten op het gedrag en de oestrus cyclus zichtbaar, terwijl het verschil tussen plasma concentraties van deze ratten en de humane situatie op dit moment slechts een orde van grootte is.

Samenvattend kan worden geconcludeerd dat continue blootstelling aan verbindingen zoals PBDEs, PCBs en hun metabolieten, maar ook gehalogeneerde fenolen, die een mogelijk endocrien verstorende werking bezitten (bijv. schildklierhormoon-achtig of oestrogeen), een bedreiging kan vormen voor de ontwikkeling van het kind. Hoewel PBDEs minder effecten laten zien wat betreft bijv. de schildklierhormoon-niveaus (Zhou *et al.*, 2001) in knaagdieren ten opzichte van coplanaire PCBs (Seo *et al.*, 1995), is het mogelijk dat de effecten in totaal mogen worden opgeteld. Voorlopige resultaten gepubliceerd door Hallgren en Darnerud (1998) tonen aan dat er inderdaad enig bewijs is

voor een additief effect wat betreft de verlaging van T_4 niveaus bij gelijktijdige toediening van PBDEs en PCBs (Aroclor 1254) of gechloreerde paraffine. Het is derhalve noodzakelijk dat bij volgend onderzoek dat gericht is op het vinden van associaties tussen blootstelling aan organohalogene verbindingen en de effecten op de humane ontwikkeling niet alleen de uitgangsstoffen maar juist ook de fenolische metabolieten worden meegenomen in de analyses. In geval van endocrien actieve stoffen is het waarschijnlijk dat zwangere vrouwen een extra risico lopen voor mogelijke hormoonverstoring omdat de ontwikkelende foetus extra gevoelig is voor kleine veranderingen in bijv. schildklierhormoonniveaus. Een extra risicofactor waaraan gedacht moet worden zijn vrouwen met een jodium deficiëntie. Ongeveer twaalf procent van de algemene bevolking en ongeveer 15 procent van de vrouwen in een leeftijd waarop ze kinderen kunnen krijgen zijn jodium deficiënt (in de Verenigde Staten, Hollowell *et al.*, 1998). Het is daarom van groot belang dat het testen van stoffen op hun mogelijke endocriene activiteit in een eerder stadium van de productontwikkeling wordt uitgevoerd om zo de verspreiding van dit soort stoffen in het milieu te kunnen voorkomen.

Abbreviations

2,4-DBP	2,4-dibromophenol
3,5-T_2	3,5-diiodothyronine
4-OH-CB107	4-hydroxy-2,3,3',4',5-pentachlorobiphenyl
4-OH-DE	4-phenoxyphenol
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	5-hydroxytryptamine
AGD	anogenital distance
AhR	arylhydrocarbon receptor
β-NF	β-Naphthoflavone
BAEPs	brain stem auditory evoked potentials
BPA	bisphenol A
CALUX	chemical activated luciferase gene expression
CL	corpora lutea
CN	caudate nucleus
CLOF	clofibrate
CRL	crown-rump length
CYP450	cytochrome P450
DA	dopamine
D-II	type II 5'-thyroxine deiodinase
DDT	2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane
DecaBDE	decabromodiphenyl ether
DiBBPA	dibromobisphenol A
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
DRE	dioxin response elements
E_2	estradiol
EC_{50}	concentration causing 50% of the maximum effect
EINECS	European Inventory of Existing Chemical Substances
E/P-ratio	estradiol/progesterone ratio
ER	estrogen receptor
EROD	ethoxyresorufin-*O*-deethylase
FC	frontal cortex
FT_4	free thyroxine
GD	gestation day
HCB	hexachlorobenzene
hEST	human estrogen sulfotransferases
HPT-axis	hypothalamus-pituitary-thyroid axis
HVA	homovanillic acid
IC_{50}	concentration causing 50% inhibition
ID-1	type I iodothyronine deiodinase
IPCS	International Programme on Chemical Safety

K_a	binding affinity constant
LOT	lateral olfactory tract
MeO	methoxylated
MBBPA	monobromobisphenol A
NA	nucleus accumbens
NE	norepinephrine
OctaBDE	octabromodiphenyl ether
OECD	Organisation for Economic Co-operation
OH-	hydroxylated
PB	phenobarbital
PBBs	polybrominated biphenyls
PBDEs	polybrominated diphenyl ethers
PBP	pentabromophenol
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PCP	pentachlorophenol
pentaBDE	pentabromodiphenyl ether
PFC	prefrontal cortex
PHAHs	polyhalogenated aromatic hydrocarbons
PND	postnatal day
POPs	persistent organic pollutants
ppm	parts per million
PROD	pentoxyresorufin-*O*-deethylase
PVC	persistent vaginal cornification
RENCO	Risk of endocrine contaminants (EU-project)
RBP	retinol binding protein
SNB	spinal nucleus of the Bulbocavernosus
SPL	sound pressure levels
T_2-like OH-BDE	4-(2,4,6-tribromophenoxy)phenol
T_3-like OH-BDE	2-bromo-4-(2,4,6-tribromophenoxy)phenol
T_4-like OH-BDE	2,6-dibromo-4-(2,4,6-tribromophenoxy)phenol
T_3	triiodothyronine
T_4	tetraiodothyronine
TBP	2,4,6-tribromophenol
TCBPA	tetrachlorobisphenol A
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TBBPA	tetrabromobisphenol A
TriBBPA	tribromobisphenol A
TetraBDE	tetrabromodiphenyl ether
TSH	thyroid stimulating hormone
TTR	transthyretin
UDP-GT	uridine diphosphoglucuronosyl transferase activity

Curriculum vitae

Ilonka Arnoldina Theodora Maria Meerts was born in Venlo, the Netherlands, on June 7, 1972. In June 1990 she graduated from the Rijksscholengemeenschap Den Hulster in Venlo and in September of the same year she started to study Molecular Sciences at the Wageningen Agricultural University (now: Wageningen University, WU), the Netherlands, with a Chemical and Biological specialization. During this study she performed research at the department of Molecular Genetics (WU; supervision: Dr. Ir. J.H.M. Lammers and Prof. Dr. C. Heyting), the department of Anthropogenetics (Academic Medical Centre, Amsterdam; supervision: Dr. J.M.N. Hoovers), the department of Medical Oncology (University Hospital Nijmegen; supervision: Drs. Y.L.M. Kamm and Prof. Dr. Ir. I.M.C.M. Rietjens) and the department of Pediatrics (University Hospital Nijmegen; supervision: Dr. M. Huizing and Dr. W. Ruitenbeek). In January 1996 she graduated for her M.Sc. in Molecular Sciences. From May 1996 until May 2000 she worked as a Ph.D. student at the Department of Toxicology of the Wageningen University on a collaborative study on the possible adverse effects of exposure to organohalogen compounds on human health, financed by the European Committee, under supervision of Prof. Dr. A. Brouwer. During her Ph.D. she also attended several courses of the Postdoctoral training in Toxicology. Since May 2000 she is working as a study director at the Department of Genetic & Ecotoxicology of NOTOX Safety and Environmental Research B.V. in 's-Hertogenbosch, the Netherlands.

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Dankwoord

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