The Neuroendocrine Effects of Non-Dioxin-Like PCBs: Filling in the Gap

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The Neuroendocrine Effects of Non-Dioxin-Like PCBs: Filling in the Gap

Een Beter Begrip van de Neuro-Endocriene Effecten van Niet-Dioxine-Achtige PCBs

(met een samenvatting in het Nederlands)

Proefschrift

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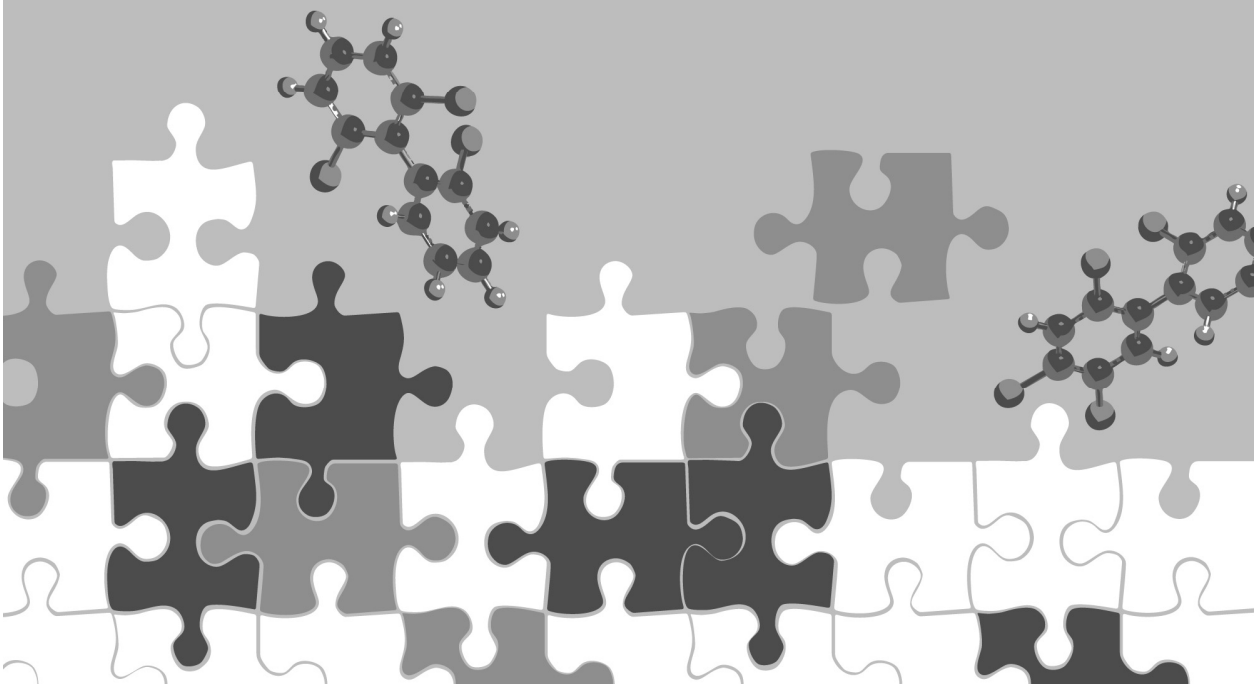
Table of contents

List of Abbreviations

AR – Androgen Receptor
AhR – Aryl hydrocarbon receptor
BFRs – Brominated flame ratardants
Bw – Body weight
cAMP – Cyclic adenosine monophosphate
CHO – Chinese hamster ovary cell line
cGMP – Cyclic guanosine monophosphate
CYP – Cytochrome P450
DL-PCBs – Dioxin-like-PCBs
EFSA – European Food Safety Authority
ERα – Estrogen receptor alpha
ERβ – Estrogen receptor beta
$GABA_A$ R – $GABA_A$ receptor
GABA – γ-Aminobutyric acid
GR – Glucocorticoid receptor
GSH – Glutathione
H295R – Human adrenocorticocarcinoma cell line
HPAG – Hypothalamic-pituitary-adrenal-gonadal axis
LOAELs – Lowest observed adverse effect levels
MCF-7 – Human mammary adenocarcinoma cell line
$MeSO_2$-PCBs – Methylsulfony-PCBs
MOE – Margin of exposure
NE – No effect
NOAELs – No observed adverse effect levels
NOS – Nitric Oxide Synthase
OD – Optical density
OH-PCBs – Hydroxylated PCBs
PBDEs – Polybrominateddiphenyl ethers
PC12 – Rat pheochromocytoma cell line
PCB – Polychlorinated biphenyl
PGE2 – Prostagladin E2
PKC – Protein kinase C
PMA – Phorbol 12-myristate 13-acetate
POPs – Persistent organic pollutants
PPB – Parts per billion
PPM – Parts per million
RyR – Ryanodine receptor
SAR – Structure activity relationship
TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin
WHO – World Health Organization

Chapter 1

General introduction



1. Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are a group of synthetic organic chemicals, which were first commercially produced in the late 1920's. Due to their physical and chemical properties, including non-flammability, chemical stability and low conductivity, PCBs have been widely used in a number of industrial and commercial applications. Their production and commercialization was gradually stopped between 1970's and 1980's in most western countries. At the Stockholm convention in 2001, these compounds were scheduled for further elimination until 2028. It is estimated that since the 1980's approximately 1.7 million tons of PCBs in technical mixtures have been produced, from which approximately 600 000 tons in USA and approximately 400 000 tons in Europe (for review see (Breivik *et al.*, 2002)).

1.1. Dioxin-like PCBs and non-dioxin-like PCBs

There are 209 theoretical PCB congeners that differ in the number of chlorination and chlorination pattern of each phenyl ring. Chlorination in the positions 3, 3', 5, 5' are named "*meta*", while the positions 4, 4' and 2, 2',6, 6' are referred to as "*para*" and "*ortho*", respectively. Figure 1 shows the structural formula of PCBs and the numbering of the carbon atoms in the two rings.

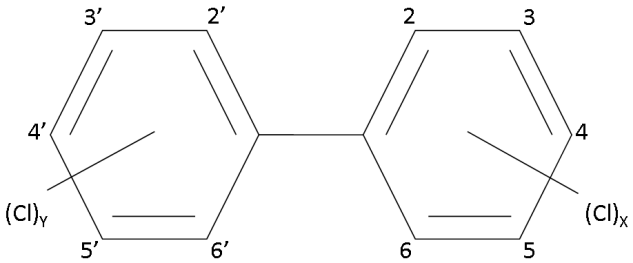


Figure 1. Chemical structure of PCBs and numbering of carbons where chlorine substitution may occur.

If chlorine substitution occurs exclusively in the *meta*- and *para*-positions, PCBs assume a planar configuration. There are 12 different congeners with this planar structure, which are often referred to as dioxin-like-PCBs (DL-PCBs). The DL-PCBs have been extensively studied and their toxic and biological effects are mainly associated with binding and activation of the aryl hydrocarbon receptor (AhR) transduction pathway. As such, they have been assigned a international toxic equivalent factor relating their toxicity to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Van den Berg *et al.*, 1998, Van den Berg *et al.*, 2006). Non-dioxin-like-PCBs (NDL-PCBs), also referred to as *ortho*-PCBs, have a 1-4 chlorine substitution on the *ortho*-positions. This substitution is translated into a non-planar spatial orientation, which impairs interaction with the AhR that is essential for dioxin like effects. Figure 2

shows the planar spatial orientation of DL-PCB126, and the non-planar spatial orientation of NDL-PCB180 (cf. (A) and (B)). NDL-PCBs present a different toxicological profile, among other having neurotoxic and endocrine disruptive potentials.

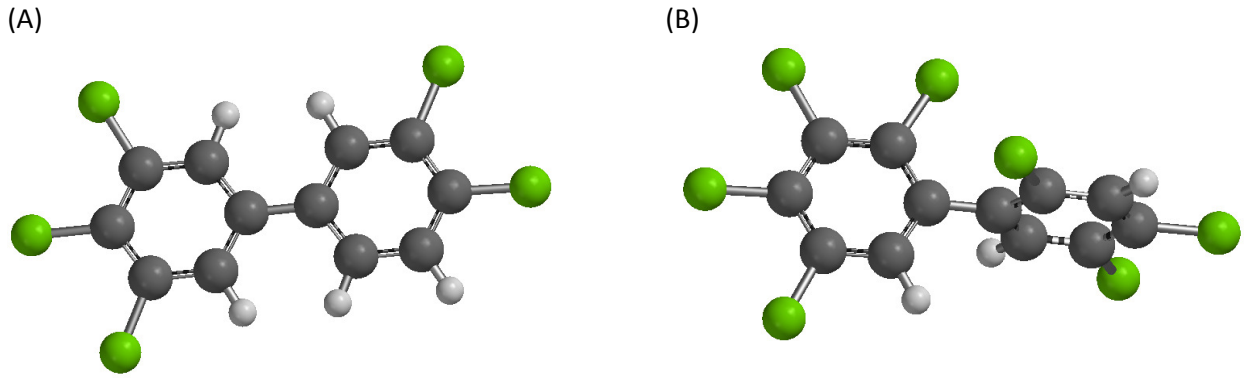


Figure 2. The spatial orientation of DL-PCB126 (A) and NDL-PCB180 (B). Carbon atoms on the phenyl ring are represented as dark grey spheres, whereas hydrogens are represented in light grey spheres. The green spheres represent the chlorine substitution on the phenyl ring.

2. Environmental levels

Due to the ban on production and commercial use of PCBs, environmental levels of these persistent organic pollutants (POPs) have slowly decreased over the last decades. However, due to their environmental persistence these can still be found in the environment and human samples.

2.1. PCB metabolism and distribution

PCBs are organic pollutants that bioaccumulate and biomagnify in the (human) foodchain. However, these can also be metabolized by phase I enzymes into hydroxylated PCBs (OH-PCBs) and by phase II enzymes into e.g. methylsulfony-PCBs ($MeSO_2$-PCBs). The first step in PCB metabolization (oxidation) mainly involves phase I enzymes of the cytochrome P450 (CYP) family and occurs via epoxide formation or by direct insertion of a hydroxyl group. Arene oxides are reactive intermediates that can further form dihydrodiol-PCBs, hydroxyl-PCBs (OH-PCBs), glutathione (GSH) conjugates or even adducts to DNA, proteins or lipids (Letcher *et al.*, 2000, Safe, 1993). Although the majority of OH-PCBs is excreted either in feces or urine, these can still be found in blood of humans and wildlife, mainly bound to proteins. Further, due to their lower lipophilicity compared to parent compounds, these do not partition to the lipid fraction efficiently (Malmberg *et al.*, 2004). However, these PCB metabolites can easily cross the blood placenta barrier at a higher rate than the

parent compounds (Soechitram *et al.*, 2004). As a result of these metabolic and kinetic processes, OH-PCBs constitute 26% of all PCBs found in maternal blood. This percentage doubles (53%) for e.g. cord blood plasma and levels can be as high as 120 and 88 pg/g fresh weight, respectively (Guvenius *et al.*, 2003). Thus OH-PCBs can easily cross the blood placental barrier and/or may be formed on the fetal side.

Further OH-PCB metabolism may also include phase II enzymes in which in a multistep pathway involving GSH conjugation, mercapturic acid pathway degradation, enterohepatic circulation, methylation and further oxidation occurs (Letcher *et al.*, 2000, Bakke *et al.*, 1982). As a result, e.g. $MeSO_2$-PCBs have a lipophilicity only slightly lower than their parent compounds explaining the easy lipid partition of these secondary metabolites (Letcher *et al.*, 2000, Hovander *et al.*, 2006). Thus, $MeSO_2$-PCBs are found at relatively low concentrations in blood, but due to their lipophilicity and specific protein binding they accumulate in a high tissue-specific manner. Predominantly these are found in liver, lungs and breast milk.

Further, PCB metabolism and subsequent elimination is highly dependent on the species and degree or pattern of chlorination (Tanabe *et al.*, 1981, McKinney *et al.*, 2006). Higher chlorinated congeners are metabolized with more difficulty than PCBs with less chlorine atoms. In addition, PCBs with vicinal non-substituted carbons are more readily eliminated. The half-lives of the six most common NDL-PCBs in humans and in rats, as reported in literature, are given in table 1.

Table 1. Half-lives of the six most commonly found NDL-PCBs in humans and in rats (adapted from EFSA 2005).

PCB	T½ (Days - Years) Humans	Ref	T½ (Days) Rat	Ref
28	18/44 days [a)] 182 days 16.8 months 4.8 years 3.0 years	(Luotamo *et al.*, 1991) (Wolff and Schecter, 1991) (Brown *et al.*, 1994) (Wolff *et al.*, 1992) (Yakushiji *et al.*, 1984)	1.4/6 [b)]	(Tanabe *et al.*, 1981)
52	5.5 years[c)]	(Wolff *et al.*, 1992)	0.9/3.4	(Tanabe *et al.*, 1981)
101	7/14 days 5.7 years[d)]	(Luotamo *et al.*, 1991) (Wolff *et al.*, 1992)	2.6/35	(Tanabe *et al.*, 1981)
138	10.7 months 3.4 years 6-7 years 16.3 years 16.7 years 20/32 years	(Buhler *et al.*, 1988) (Ryan *et al.*, 1993) (Brown *et al.*, 1994) (Yakushiji *et al.*, 1984) (Wolff *et al.*, 1992) (Yakushiji *et al.*, 1984)	101 >90	(Oberg *et al.*, 2002) (Tanabe *et al.*, 1981)
153	11.3 months 3.9 years 12.4 years 26/47 years 27 years	(Buhler *et al.*, 1988) (Ryan *et al.*, 1993) (Brown *et al.*, 1994) (Chen *et al.*, 1982) (Yakushiji *et al.*, 1984)	113 >90	(Oberg *et al.*, 2002) (Tanabe *et al.*, 1981)
180	4.1 months 4.8 years 9.9 years	(Buhler *et al.*, 1988) (Ryan *et al.*, 1993) (Wolff *et al.*, 1992)	81 >90	(Oberg *et al.*, 2002) (Tanabe *et al.*, 1981)

a) Two values given in the study depending on sample selections
b) The two values presented represent first and second phase half-lives for compounds with biphasic eliminations.
c) Co-elution between PCB 47, 49 and 52
d) Co-elution between PCB 99 and 101

3. Human exposure

Bioaccumulation and biomagnification of PCBs, both DL and NDL congeners, account significantly for human exposure to these pollutants. The most common congeners in food and feed are NDL-PCBs. In addition, the main route of exposure is food, which accounts for more than 90% of human exposure to NDL-PCBs. For background exposure this has been estimated to be 10-45 ng/ kg bw.day for NDL-PCBs. Young children, however, are exposed to significant higher concentrations of NDL-PCBs and, even excluding breastfeeding, levels of NDL-PCBs can be 27-50 ng/kg bw. Further, in specific cohorts comparing exposure of adults and children (up to six years old), there is a higher exposure of children, which can go up to 2.5-fold difference (European Food Safety Authority (EFSA), 2005).

Human exposure to NDL-PCBs is dominated by higher chlorinated PCB138, 153 and 180, since these are the most abundant congeners in food, like meat, fish and dairy products (Juan *et al.*, 2002, Szlinder-Richert *et al.*, 2009). However, for lower chlorinated and volatile congeners (mainly PCB28 and PCB52) inhalation of contaminated indoor air can also account for a significant route of exposure (Norstrom *et al.*, 2010). Air concentration ratios of lower/higher chlorinated NDL-PCBS are higher than in other matrices, like food or soil. Therefore, indoor air exposure may account for an increase in the presence of more volatile congeners present in blood. However, it should be noticed that the ratio of exposure is not found in the same proportions in blood and serum. This may be explained by a faster metabolization of these lower chlorinated congeners.

Recently, the contribution of other routes of PCB exposure has been taken into consideration. House dust, and its contribution to the total adult human exposure to structurally similar polybrominateddiphenyl ethers (PBDEs), has been found to be of equal importance as food (Dirtu and Covaci, 2010). Although in some studies the same contribution could also be established for adult human exposure to NDL-PCBs, the results are mainly dependent on the geographic sampling (Harrad *et al.*, 2009). Most importantly, exposure to NDL-PCBs via house dust becomes of more concern for children and toddlers (Harrad *et al.*, 2009). Interestingly, it has been suggested that inhalation of house dust is mainly responsible for exposure to lower-chlorinated NDL-PCBs, whereas ingestion contributes proportionally more to the uptake of higher molecular weight and generally less easily metabolized congeners (Thomas *et al.*, 2006).

Fetuses, breast-fed infants and toddlers are undergoing rapid growth. Therefore, perinatal and young age exposure to PCBs (and metabolites) should be taken into account in risk assessment, since it may affect developmental processes including development of the nervous, endocrine and reproductive systems as well as several other organ systems.

The neuroendocrine system incorporates interactions of hormones produced and secreted in different glands, which can closely control processes in other organs. For example, brain sexual differentiation is an important process that is under the close control of hormonal exposure. Although the brain is capable of producing estradiol via aromatase, the levels of circulating hormones influence the activity and expression steroidogenic enzymes as well as activation of hormone receptors. Importantly, changes in hormonal homeostasis during the development stage can have permanent effects, interfering not only with the reproductive system, but might also (indirectly) induce neurobehavioral effects.

4. Toxic effects of NDL-PCBs

PCB exposure has been linked with many adverse health effects, such asthose on the thyroid and immune system, but maybe most concerning are effects on neurobehavioral development and reproductive functions. However, these studies often do not differentiate between DL and NDL congeners as they occur concomitantly. As NDL-PCBs account for the majority of PCBs found in food and human tissues, it is important to have a better understanding of their toxicity. Many experimental *in vivo* and *in vitro* studies have shown that NDL-PCBs are capable of interfering with the development of the reproductive and neurological system, effects which are also well known from the DL-PCBs.

4.1. Neurobehavioural effects

Several epidemiological studies have identified deficits in learning and memory as a result of adult environmental exposure to PCBs via food (Schantz *et al.*, 2001). In deceased Alzheimer patients, NDL-PCBs in brain were found at a higher level than in control individuals (Corrigan *et al.*, 1998). Further, Seegal and co-workers reported a higher incidence of Parkinson's disease in former capacitor production workers who have been occupationally exposed to high levels of PCBs (Seegal, 2004). The incidence of neurobehavioural effects as a result of NDL-PCB exposure is dependent on the window of exposure, i.e. the life stage involved.

In utero and lactational exposure of foetus and children born to mothers with high blood and milk PCB levels, showed lower IQ scores, increased behavioral disorders and delayed motor development (Schantz *et al.*, 1996, Rogan *et al.*, 1986, Jacobson and Jacobson, 1996, Vreugdenhil *et al.*, 2004b).

Interestingly, some effects could be related to the breastfeeding (in comparison to formula feeding). Further, follow up studies indicated that prenatal exposure to NDL-PCBs is also associated with lower scores at cognitive ability tests (Vreugdenhil *et al.*, 2004b, Vreugdenhil *et al.*, 2004a). Epidemiological and experimental studies also indicated that exposure to PCBs *in utero* and through breastfeeding, induce neurodevelopmental and neurobehavioral effects in children and animal offspring. Importantly, these effects turned out to be persistent and could still be detected in adults (Faroon *et al.*, 2001, Winneke *et al.*, 1998).

4.1.1. In vivo

Most *in vivo* studies have used complex commercial mixtures (e.g. Arochlors) of DL- and/or NDL- PCBs. However, it was soon clear that effects were dependent on the composition of these mixtures. Consequently, single congeners were investigated in more detail and perinatal exposure to DL-PCBs, such as PCB77, PCB118, PCB126 or

PCB156, induced changes in sweet preference in female rats, in overall activity and in learning and memory measured by the radial arm maze (Schantz *et al.*, 1996, Amin *et al.*, 2000, Agrawal *et al.*, 1981). Experiments conducted with NDL-congeners, such as PCB28, PCB52, PCB95 or PCB153, showed animals with deficits in spatial learning, memory and habituation of activity (Schantz *et al.*, 1995, Eriksson and Fredriksson, 1996). More recently, Boix *et al.*, (2010) and Piedrafita *et al.,* (Piedrafita *et al.*, 2008a, Piedrafita *et al.*, 2008b) have shown that perinatal exposure to NDL-PCBs, such as PCB52, PCB138 or PCB153 impair conditional task learning, motor coordination, spontaneous motor activity, vertical activity and jumping in male and females. Taken together, all these experimental studies indicate that NDL-PCBs may have comparable overall effects as DL-PCBs, albeit at higher dose levels.

4.1.2. In vitro

In vitro studies have been performed to elucidate the neurodevelopmental and neurobehavioural effects seen *in vivo*. Some of the *in vivo* effects can be explained by changes in the dopaminergic system (Fonnum *et al.*, 2006, Seegal *et al.*, 2002). This is further corroborated by *in vitro* work, in which exposure to PCBs decreases dopamine levels in rat pheochromocytoma cells (PC12 cells), a neuroendocrine *in vitro* model often used to study neurotoxicity (Angus and Contreras, 1996, Angus *et al.*, 1997). The exact mechanism underlying the decrease in intracellular dopamine is not clear, but could involve an inhibition of tyrosine hydroxylase, dopamine decarboxylase, vesicular uptake of dopamine or plasma membrane uptake of dopamine. Further, NDL-PCBs are also pointed out to interfere with transport of neurotransmitters in synaptosomes, such as glutamate and GABA, which could result in a longer activation of neurotransmitter receptors. *Ortho*-chlorinated PCBs with four to five chlorine substituents were found to be the congeners showing the strongest inhibition of neurotransmitter transport (Mariussen and Fonnum, 2001)

As calcium homeostasis is critical for cellular function, many studies have focused on this parameter and tried to identify how PCBs can interfere with calcium signalling. PCBs were able to affect calcium uptake by synaptosomes (Kodavanti, 2006). This may be explained by interference with activation of voltage-gated calcium channels, glutamate receptors or uptake into intracellular calcium stores. Further, calcium homeostasis can be regulated by the ryanodine receptor (RyR) complex and it has been shown that RyR activation by PCBs is congener dependent (Pessah *et al.*, 2006). Disruption of intracellular calcium levels may also be responsible for induction of oxidative stress, explaining PCB-induced cell death (Fonnum and Mariussen, 2009). This can occur via different pathways, such as protein kinase C activation (Kodavanti *et al.*, 1994), phospholipase 2 activation (Kodavanti and Derr-Yellin, 2002), nitric

oxide synthase (NOS) (Kang *et al.*, 2002) and glutamate receptor activation (Mariussen *et al.*, 2002).

4.2. Reproductive and developmental effects

Most information regarding human exposure to PCBs refers to populations living in highly polluted areas where higher incidences of menstrual irregularities and miscarriages were reported in women (Kusuda, 1971, Gerhard *et al.*, 1998). In addition, maternal and paternal exposure has been associated with an increased sex ratio (number of boys/number of girls) (Weisskopf *et al.*, 2003) and lower body weight of newborns (Taylor *et al.*, 1989). In men, a higher PCB serum concentration has been related to infertility and decreased sperm mobility (Pines *et al.*, 1987, Bonde *et al.*, 2008).

4.2.1. In vivo

Several *in vivo* studies have focused on the endocrine effects of commerical or reconstituted mixtures. The main effects were seen in rodents that had been exposed perinatally. Hany *et al.*, (1999) showed that perinatal exposure to DL- and NDL-PCBs resulted in female offspring with elevated uterine weights and adult males with reduced testes weights and testosterone serum levels. Further, they showed feminization of the male offspring in a sweet preference test, indicating long-lasting changes in neuronal brain organization. In a follow-up study, Kaya *et al.*, (2002) reported dose-dependent changes in sex hormone concentration in weanling female and male adults. Perinatal exposure of rats to a mixture of high-chlorinated (common) NDL-PCBs and DL-PCB126, resulted in decreased body weight of male and female offspring at weaning and earlier onset of puberty in females and delayed testicular descent in males. Apparently, the effects were sex-dependent, since males showed a delay in copulatory behaviour, whereas females behaved normally (Cocchi *et al.*, 2009). The treatment also affected hypothalamic expression of enzymes important for steroidogenesis, such as aromatase and 5α-reductase1. Up- or down-regulation of these enzymes was dependent on the sex of the offspring; aromatase was strongly up-regulated in males but not in females, whereas 5α-reductase1 expression was inhibited mainly in females (Colciago *et al.*, 2009).

There are several studies in which individual PCB congeners were administrated to animals in order to elucidate the effect of each congener on reproductive and developmental end-points. As a result of perinatal exposure to DL-PCBs, such as PCB77, PCB118, PCB126 or PCB169, offspring showed changed in anogenital distance, time to vaginal opening or decreased birth weights. Some of these effects were persistent into adulthood and could still be found in the second generation (Schantz *et al.*, 1995, Faqi *et al.*, 1998, Seegal *et al.*, 1997, Ness *et al.*,

1993). Focusing on NDL-PCBs, such as PCB28, PCB47, PCB95 or PCB153, perinatal exposure caused reduced fetal, nursing and weaning weight in pups (Schantz *et al.*, 1995, Ness *et al.*, 1993). Comparison of No Observed Adverse Effect Levels (NOAELs) and Lowest Observed Adverse Effect Levels (LOAELs) of the different studies indicated, that DL-PCBs are more potent than NDL-PCBs. LOAELs and NOAELs of DL-PCBs, with PCB126 being the most potent, are in the sub μg/kg/day, while these of the common NDL-PCBs, with PCB153 being the most potent one, are in the high mg/kg/day range. In general, these results indicate that there is a quantitative difference in reproductive and developmental toxicity between DL and NDL-PCB that spans many order orders of magnitude.

4.2.2. *In vitro* – (anti-) estrogenic actions and receptor interaction

In vivo studies clearly indicate that NDL-PCBs can act as endocrine disruptors that can explain the reproductive and developmental effects. Therefore, it is important to understand the mechanistic properties of the endocrine disruptive potential of these PCBs.

The World Health Organization (WHO) defines an endocrine disruptive compound as *"an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations"* (The International Program on Chemical Safety (IPCS), 2002).

Endocrine disruption can involve different mechanisms like direct interaction with steroid hormone receptors or the steroidogenic pathway. In receptor interactions, the compound can block (antagonist) or induce (agonist) receptor-cell signalling pathways and, for example, alter the feedback mechanisms that regulate hormone synthesis. This can potentially lead to an induced or reduced hormone synthesis with subsequent effects on the reproduction or development. Important sex hormone receptors are the androgen receptor (AR) and estrogen receptors (ERs). The latter is one of the best studied sex steroid receptors. There are two subtypes of ER, alpha (ERα) and beta (ERβ), each controlling different functions depending on the tissue where these are expressed. *In vitro*, PCBs and their OH-metabolites have been shown to bind to both ERα and ERβ (Korach *et al.*, 1988, Matthews and Zacharewski, 2000, Kester *et al.*, 2000). Some PCBs and their metabolites are also found to be estrogenic in the MCF-7 (human mammary adenocarcinoma cell line) proliferation assay. (Andersson *et al.*, 1999, DeCastro *et al.*, 2006). Interestingly the estrogenic potency was dependent on the chlorination pattern.

Endocrine disruption can also arise from direct catalytic interaction with enzymes involved in the steroidogenic pathway, or their expression regulated via the hypothalamic-pituitary-adrenal-gonadal (HPAG) axis. This axis is responsible for the regulation of steroidogenisis and consequently the production of different types of

steroid hormones: glucocorticoids (e.g. cortisol), mineralocorticoids (e.g. alsosterone), progestins (e.g. progesterone), androgens (e.g. testosterone) and estrogens (e.g. estradiol).

During the reproductive life stage, ovaries and testis are the main source of estrogens and androgens, respectively. Estrogen production is the final step of steroidogenesis, which involves aromatization of androgens by aromatase (CYP19) (Simpson, 2002). Aromatase is present in glands producing steroids (estrogens), such as ovaries, testis, adrenal glands and placenta, but also in other tissues, such as adipose tissue, brain and mammary gland (Simpson, 2002). Aromatase expression is tissue- and promoter-specific. In adipose tissue, aromatase regulation involves promoter region I.4, which is regulated by glucocorticoids; whereas in brain, gene expression is mainly dependent of promoter region I.f., inducible by androgens, phorbol esters and dibutyryl cyclic guanosine monophosphate (cGMP) (Simpson, 2004).

During fetal development the glands constituting the HPAG axis are undergoing morphological and/or functional changes and maturation is attained after birth. In foetuses, adrenals are under strong regulation of the placenta, constituting the so-called (mother-) fetal-placental unit. This unit is responsible for maturation of fetal organs necessary for extrauterine life (lungs, liver, gut), but also for intrauterine homeostasis of the estrogen synthesizing system. This plays an important role in the maintenance of pregnancy, foetal maturation and development and, in some species, the initiation of parturition (Ishimoto and Jaffe, 2010). After birth, the main function of the adrenal cortex is to synthesize and secrete androgens and glucocorticoids (Tegethoff *et al.*, 2009). Changes in the steroidogenic axis during development might result in altered circulating hormone levels, which in turn can lead to persistent changes in gene expression of steroidogenic enzymes and receptors in the HPAG axis.

Exposure to PCBs has been shown to alter the activity and expression of several steroidogenic enzymes, such as aromatase (Li, 2007, Woodhouse and Cooke, 2004, Heneweer *et al.*, 2005). The H295R adrenocorticocarcinoma cell line is a commonly used *in vitro* model to study steroidogenic interactions. Using this model, Xu *et al.*, (2006) have shown that NDL-PCBs and their $MeSO_2$-metabolits can affect multiple enzymes involved in steroidogenesis, including CYP11B1, CYP11B2, CYP19, 3ß-HSD1, 3ß-HSD2 and 17ß-HSD.

5. Scope of this thesis

Clearly, the neurotoxic potential of NDL-PCBs has been associated with neurobehavioral effects, including changes in motor activity, learning, memory and attention, which have been associated with neurotransmitter systems. So far, much

research has been focused on presynaptic mechanisms, such as neurotransmitter levels and calcium homestasis. However, there is a lack of knowledge on mechanistic information regarding postsynaptic neurotransmitter receptors.
Endocrine toxicity of NDL-PCBs involves among others androgenic and estrogenic interactions, which have been related to sex-dependent behavior and morphological changes in gonads, adrenal glands and brain. However, there is a signifcant lack of information on how individual congeners interact with steroidogenic enzymes and endocrine receptors and their mechanism of action.
Therefore, the main purpose of this thesis is to gain further insight in the neurotoxic and endocrine disruptive potential of these NDL-PCBs.

Chapters 2 and **3** focus on the potential neurotoxicity of NDL-PCBs and their effects on the postsynaptic human $GABA_A$ receptor.

In 2005 the EFSA has identified six common NDL-PCBs which alone represent approximately 50% of all NDL-PCBs present in food. However, due to the limited number of *in vivo* and *in vitro* studies performed with NDL-PCBs, risk characterization and assessment is hampered. Further, direct and acute effects of NDL-PCBs on specific neurotransmitter receptors had not been described uptil the beginning of this thesis. Therefore, in **chapter 2** the effects of six common NDL-congeners on the $GABA_A$ receptor were investigated. Further, because human exposure is not restricted to single congeners, the effects of binary mixtures of NDL-PCBs on the $GABA_A$ receptor were also studied.

The limited number of congeners studied in **chapter 2** impaired the identification of a SAR of NDL-PCBs on the $GABA_A$ receptor. Therefore, the set of NDL-PCBs used in **chapter 3** was enlarged, including an extra DL-congener as well. The choice of the used congeners was based on their chemical and physical properties, environmental abundance and toxicological activities. Further, the binary mixtures used in this chapter aimed at assessing the effects not only on potentiation, but also on activation of the receptor.

Experimental *in vivo* results have described sex dependent endocrine disruptive potential of NDL-PCBs. Further, the effects were apparently dependent on the time of exposure. However, many of these studies were performed with (reconstituted) mixtures and the purity of the used NDL-congeners was not always assured. Taken together, the endocrine disruptive effects seen could partially be attributed to the presence of DL-PCBs, known to interfere with the steroidogenic pathway and showing endocrine and reproductive disruptive potential. The brain, adrenals and gonads constitute an important axis in maintaining sex steroid homeostasis and regulating steroidogenesis during perinatal development and adult life. In **chapter 4** the effects of perinatal exposure to two commonly found NDL-PCBs on aromatase activity on gonadal and adrenal microsomes at various life-stages were investigated. Moreover, gene expression of aromatase and sex steroid receptor expression in adult rat brain were also assessed.

Traditional risk assessment of potential endocrine-disruptive pollutants, including PCBs, focus mainly on the effects of parent compounds. Still, biotransformation results in systemic exposure to PCBs and their bioactive metabolites. In **chapter 5,** a more mechanistic approach of the effects of twenty NDL-PCBs was taken by studying aromatase activity in human placental microsomes and in the H295R cell line. In addition, the interaction of twenty NDL-PCBs with the glucocorticoid receptor (GR) was studied in a recently developed yeast-based glucocorticoid receptor assay. Because high levels of OH-PCBs and $MeSO_2$-PCBs can also be found in human samples, these were studied in these assays as well.
Taken together, the data described in this thesis show the potential neuroendocrine effects of NDL-PCBs and their metabolites. Further, perinatal PCB exposure appears to cause persistent neuroendocrine developmental effects that can be detected in adults.

A summary and general discussion of the results described in this thesis are given in **chapter 6.**

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Chapter 2

Potentiation of the human $GABA_A$ receptor as a novel mode of action of lower-chlorinated non-dioxin-like PCBs

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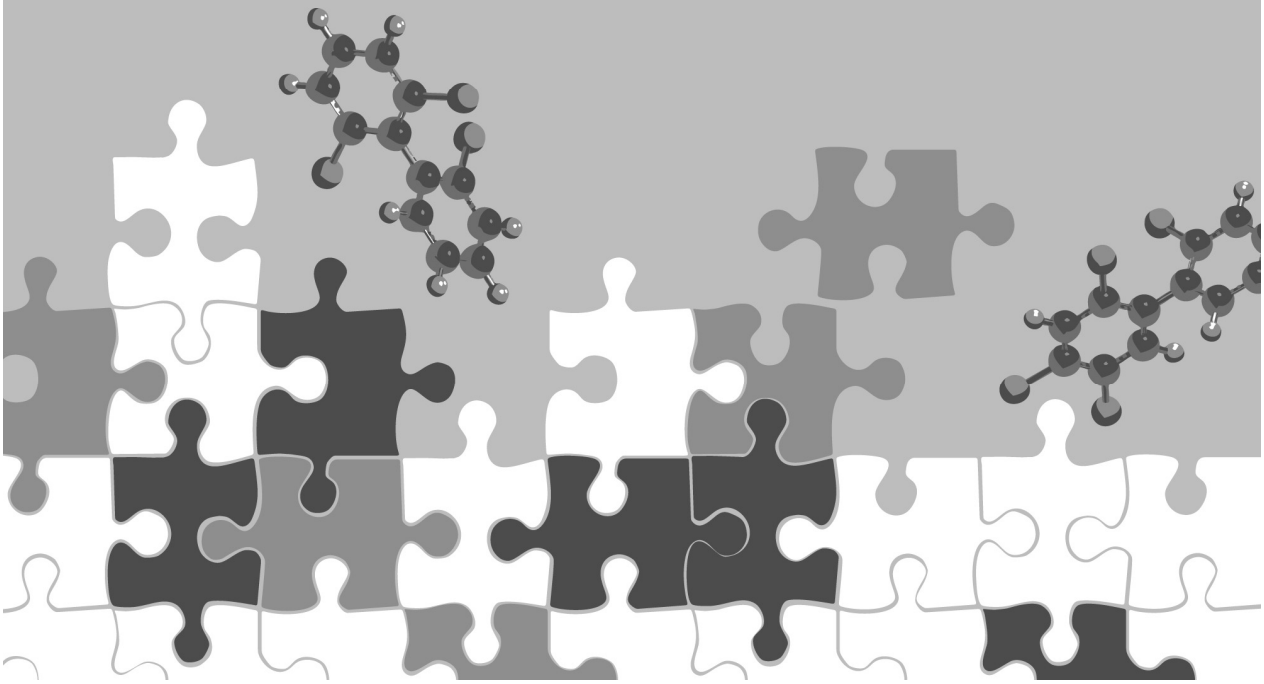

Abstract

PCBs are still ubiquitous pollutants, despite the ban on industrial and commercial use. To date, risk characterization and assessment of non-dioxin-like PCBs (NDL-PCBs), especially with respect to neurotoxicity, is hampered by a lack of data. Therefore, the effects of six common NDL congeners (PCB28, 52, 101, 138, 153 and 180) on human $GABA_A$ receptors, expressed in *Xenopus* oocytes, were investigated using the two-electrode voltage-clamp technique.

When co-applied with GABA (at EC_{20}), PCB28 and PCB52 concentration-dependently potentiate the $GABA_A$ receptor-mediated ion current. Though the LOEC for both PCB28 and PCB52 is 0.3 μM, PCB28 is more potent than PCB52 (maximum potentiation at 10 μM amounting to 98.3 ± 12.5% and 25.5 ± 1.4%, respectively). Importantly, co-application of PCB28 (0.3 μM) and PCB52 (10 μM) resulted in an apparently additive potentiation of the $GABA_A$ response, whereas co-application of PCB28 (0.3 μM) and PCB153 (10 μM) attenuated the PCB28-induced potentiation. The present results suggest that the potentiation of human $GABA_A$ receptor function is specific for lower-chlorinated NDL-PCBs and that higher molecular weight PCBs may attenuate this potentiation as result of competitive binding to human $GABA_A$ receptors. Nonetheless, this novel mode of action could (partly) underlie the previously recognized NDL-PCB-induced neurobehavioral alterations.

Introduction

Polychlorinated biphenyls (PCBs) have been widely used in numerous industrial and commercial applications (2). Although industrial production and use of PCBs has been largely prohibited in most countries since the 1980's, they are still present in electronics, plastics and building materials. Industrial and house-hold waste disposal remains the major source of PCBs in the environmental and (3) food chain (4). Food intake is one of the main routes for human PCB exposure (5), though skin contact and inhalation also provide significant exposure due to leakage of PCBs from building material into the (indoor) environment (6, 7). Regardless of the route of exposure, levels of individual PCBs in human blood have been shown to be in the low nM range (8, 9).

Based on their structural characteristics and toxicological effects, PCBs can be divided into two groups: 12 co-planar dioxin-like (DL-) PCBs and 197 non-planar non-dioxin-like (NDL-) PCBs. The DL-PCBs have been extensively studied and their toxic and biological effects are mainly associated with binding and activation of the aryl hydrocarbon receptor (AhR) transduction pathway. Contrary to the NDL-PCBs, they have been assigned a toxic equivalent factor (10) relating their toxicity to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (11). The NDL-PCBs have generally been considered less toxic because of their *ortho*-substituted chlorines, which impair interaction with the AhR (4).

Nevertheless, *in vivo* and *in vitro* studies have shown that NDL-PCBs have neurotoxic potential by interfering with intracellular signaling and disruption of Ca^{2+} homeostasis (for review see (12)). Additionally, NDL-PCBs have been shown to inhibit the uptake of dopamine, serotonin, glutamate and GABA in synaptosomes and synaptic vesicles isolated from rat brain (13, 14) and to alter neurotransmitter receptor and neurotransmitter levels in rat and mouse brain (15, 16). These alterations can possibly underlie the observed neurobehavioral effects *in vivo*, which include changes in motor activity, learning, memory and attention (16), (17), (18), (19), (20). There is a strong association of these behavioral changes with the glutamatergic and dopaminergic neurotransmitter system and especially with the GABA-ergic system as GABA is the main inhibitory neurotransmitter in the adult mammalian central nervous system and provides the main inhibitory feedback for both learning and memory and motor activity (for review see (21)). Though previous research indicated the involvement of different neurotransmitter systems, e.g., the cholinergic and GABA-ergic system in PCB-mediated neurotoxicity (16), (22), (23), acute, direct effects of PCBs on the functioning of specific neurotransmitter receptors, including dopamine, glutamate and GABA receptors, have not been described to date.

Activation of (postsynaptic) human $GABA_A$ receptors allows for influx of Cl^- resulting in hyperpolarization and a decreased activity of the neuron. GABA binds to

the pentameric $GABA_A$ receptor via two GABA-binding sites. There are, however, several other binding sites that are capable of modulating $GABA_A$ receptor function. Depending on the binding site involved, the GABA-induced Cl^- current can be potentiated or inhibited. Due to their physiological properties (anxiolytic, anticonvulsant, analgesic, sedative, etc.), benzodiazepine, ethanol and neurosteroids are among the most investigated ligands (for review see (24)). *In vitro* and *in vivo* studies have shown that the prolonged use of e.g., anesthetics or anxiolytic drugs can eventually lead to cell apoptosis in rat brain by disruption of the $GABA_A$ receptor function (10).

In 2005, the European Food and Safety Authority (EFSA) released a risk assessment publication regarding the presence of NDL-PCBs in feed and food. In this report, six abundant NDL-congeners (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) were identified as representing together ~50% of the total amount of NDL-PCBs in food (5). Despite the abundance of these congeners in food, these reference congeners have been used only in a limited number of *in vivo* and *in vitro* studies. Consequently, risk characterization and risk assessment of these relevant NDL-PCBs is hampered by a lack of data. Moreover, as specific effects of these NDL-PCBs on neurotransmitter receptors function have not been described, the aim of the present study was to investigate whether the above-mentioned six common (highly-purified) NDL-PCBs can exert acute effects on the function of the predominant receptor of the main inhibitory neurotransmitter in the mammalian brain, i.e., the $GABA_A$ receptor.

Experimental Section

Animals

All experiments were in accordance with Dutch law and approved by the Utrecht University Ethical Committee for Animal Experiments. Adult female specimen of *Xenopus laevis* frogs (provided by Dr. Wim Scheenen, Radboud University, Nijmegen, The Netherlands) were kept in copper-free water (pH 7, 1.25 mmol CaO/l, 24^{o}C) in standard aquaria (0.5 × 0.4 × 1 m; 1–15 per aquarium) with a 12 h light/dark cycle. The animals were fed earthworms three times a week (Hagens, Nijkerkerveen, The Netherlands).

Chemicals

Gabazine, GABA, neomycin solution (10 mg neomycin/ml in 0.9% NaCl), collagenase type I, NaCl and 3-aminobenzoic acid ethyl ester (MS-222) were obtained from Sigma Chemical (St. Louis, MO, USA). $CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution), $MgSO_4$, $NaHCO_3$, NaOH, $Ca(NO_3)_2$, KCl, and HEPES were purchased from Merck (Darmstadt, Germany). cDNAs of human $GABA_A$ subunits were synthesized and

provided by Paul J. Whiting (Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK).

Highly purified (>99.2%) PCBs were purchased from Neosync Inc., USA. The possible presence of PCDD/Fs and DL-PCBs impurities in the NDL-PCBs were removed by Stenberg and Andersson (Institute of Environmental Chemistry, Umeå University, Umeå, Sweden) by applying a fractionation on active carbon clean-up step. The six NDL-PCBs used were PCB28 (2,4,4'-Trichlorobiphenyl), PCB52 (2,2',5,5'-Tetrachlorobiphenyl), PCB101 (2,2',4,5,5'-Pentachlorobiphenyl), PCB138 (2,2',3,4,4',5'-Hexachlorobiphenyl), PCB153 (2,2',4,4',5,5'-Hexachlorobiphenyl) and PCB180 (2,2',3,4,4',5,5'-Heptachlorobiphenyl). PCB126 (3,3',4,4',5-Pentachlorobiphenyl) was used as DL-PCBs reference congener. PCBs and gabazine were dissolved in purity-checked dimethyl sulfoxide (DMSO), provided by Stenberg and Andersson (Institute of Environmental Chemistry, Umeå University, Umeå, Sweden). PCBs stock solutions of 25 mM were further diluted to obtain a final experimental concentration ranging from 10 nM to 10 µM. Gabazine stock solution of 100 mM was further diluted to obtain an experimental concentration of 25 µM. . DMSO at concentrations up to 0.5% (v/v) had no effect on the $GABA_A$ receptor mediated Cl^- currents. In the present experiments, the final concentration of DMSO in PCB (and gabazine)-containing saline was always kept below 0.1% (v/v).

Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ Receptors in Xenopus laevis Oocytes

All procedures are essentially as described previously (25). Briefly, female *Xenopus laevis* were anaesthetized by submersion in 0.1% MS-222 and ovarian lobes were surgically removed. Oocytes were treated with collagenase type I (1.5 mg/ml Ca^{2+} free Barth's solution) for 90 min at room temperature before manual defolliculation. cDNA coding for the human α_1, β_2, and γ_{2L} subunits of human $GABA_A$ receptors, dissolved in distilled water at a 1:1:1 molar ratio, was injected into the nuclei of stage V or VI oocytes using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA, USA). The injected volume was 23 nl/oocyte (~1 ng of each subunit). Sham-injected oocytes were injected only with 23nl of distilled-water, i.e., without cDNA. Oocytes from *Xenopus laevis* are a commonly used tool in studying direct effects plasma membrane proteins (receptors) (for review see (26)). The foreign cDNA of the receptor of choice, injected into the nucleus of the oocyte, is translated and the expected functional receptor is subsequently expressed in the membrane of the oocyte. Consequently, this system is very suitable to study direct effects of drugs and chemicals on neurotransmitter receptor function (25). Following injection of the cDNA, or only demi-water, oocytes were incubated at 21°C in modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 10 µg/ml neomycin (pH 7.6 with

NaOH). Experiments were performed on oocytes after 2-5 days of incubation. Each experiment was repeated on oocytes obtained from two or three different animals.

Electrophysiological Recording

Following translation of injected cDNA the oocytes expressed functional $GABA_A$ receptors in the membrane. Ion currents associated with $GABA_A$ receptor activity were measured with two-electrode voltage clamp techniques using a Gene Clamp 500B amplifier (Axon Instruments) with high-voltage output stage according to the methods described previously (25). Recording microelectrodes (0.5-2.5 MΩ) were filled with 3 M KCl. Oocytes, placed in a Teflon recording tube, were voltage clamped at -60 mV and continuously superfused (~30 ml/min) with saline solution, containing (in mM): 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH). Aliquots of freshly thawed stock solutions of GABA in demi-water and of the different PCBs and gabazine in DMSO were added to the saline immediately before the experiments. Oocytes were exposed to compounds by switching the perfusate from saline to PCB and/or GABA-containing saline using a servomotor-operated valve. For specific experiments, oocytes were exposed to a mixture of PCBs (0.3 µM PCB28 + 10 µM PCB52 or 0.3 µM PCB28 + 10 µM PCB153) or a mixture of PCB28 (10 µM) and gabazine (25 µM). In order to minimize absorption of PCBs to the perfusion system, glass reservoirs and Teflon tubes (PTFE; 4x6mm, Rubber, Hilversum, The Netherlands) were used. Ocytes were exposed repeatedly to different GABA- and/or PCB-containing solutions. Therefore, a washout period of 2-5 min between each application was introduced, allowing receptors to recover from desensitization. The lipophilic nature of the PCBs apparently did not affect the observed rapidly reversible potentiation of the GABA-evoked response, as repeated applications of PCBs did not enhance or attenuate the effect. Membrane currents were low-pass filtered (8-pole Bessel; 3 dB at 0.3 kHz), digitized (12 bits; 1024 samples/record), and stored on disk for computer analysis.

Data Analysis and Statistics

Peak amplitudes of GABA-induced ion currents were measured and normalized to the amplitude of GABA-induced (1 mM) control responses to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitude over time. Normalized ion currents were plotted against GABA concentration in each experiment. A GABA concentration-effect curve was fitted to the data obtained in separate experiments using Prism (Graphpad Software, La Jolla, CA, USA). The percentage of PCB-induced potentiation of the GABA-induced ion current was calculated from the quotient of the maximum amplitude of the GABA-

PCB co-application response (during 20 s) and the maximum amplitude of the control response. Data represent mean ± S.E.M of *n* oocytes.
The concentration-dependence of the potentiating effects of PCBs were determined by one-way ANOVA (p<0.05) and post-hoc Bonferroni testing. A two-tailed unpaired Student's *t*-test (p<0.05) was used to determine the effects of binary PCB-mixtures.

Results

Functional Properties of Human $GABA_A$ Receptors Expressed in Xenopus laevis Oocytes

Oocytes expressing human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, voltage-clamped at -60 mV, were exposed to saline solution containing 0.3 µM to 3 mM of GABA. Ion current amplitude increased with increasing GABA concentration and saturated around 1 mM GABA (see insets Fig. 1). Normalized GABA-induced currents were plotted against GABA concentration to obtain a concentration-effect curve and to determine EC_{20}, EC_{50} and EC_{80} values, i.e., the concentrations producing 20%, 50% and 80% of the maximal response (Fig. 1). In line with previous reports (27), (28) the concentration-effect curve was best fitted with a Hill coefficient of 1.27 (± 0.05) and EC_{20}, EC_{50} and EC_{80} values amounted to 22, 65 and 193 µM (n=10), respectively.

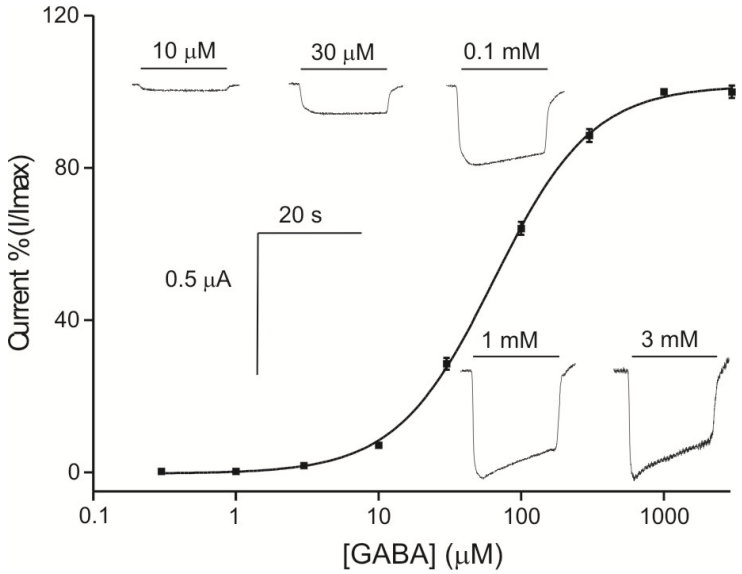

Figure 1. GABA concentration-response curve of human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. The fitted line is a Hill curve with a slope of 1.27 and an EC_{50} of 65 µM. Data are mean ± S.E.M. (n=10). Insets show example recordings of inward Cl^- currents evoked at different GABA concentrations, as indicated by the solid lines on top of the recordings.

NDL-PCBs Effects on $GABA_A$ Receptor Activation

In subsequent experiments, six common NDL-PCBs (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) and one DL-PCB (PCB126) were screened for possible antagonistic and agonistic effects on $GABA_A$ receptor activation. To assess possible agonistic effects on the human $GABA_A$ receptors, oocytes were exposed to PCB-containing saline (1 or 10 µM) for 20 s. However, at these concentrations none of the tested PCBs was able to activate $GABA_A$ receptors (not shown).

Further, co-application of these PCBs (1 µM and 10 µM) with GABA either at EC_{20} or at EC_{80} revealed that none of these PCBs had antagonistic properties (not shown). However, upon co-application of GABA at EC_{20}, but not EC_{80}, with PCB28 or PCB52 a marked potentiation of the GABA-induced ion current was observed (Fig. 2A). This potentiation appeared to be specific for lower-chlorinated NDL-PCBs as PCB101, PCB126, PCB138, PCB153 and PCB180 were unable to potentiate the GABA-induced ion current (Fig. 2A).

The potentiation of the GABA-induced ion current (at EC_{20}) by PCB28 and PCB52 was further tested at concentrations ranging from 10 nM to 10 µM. The PCB-induced potentiation was concentration-dependent (ANOVA, $p<0.05$; Fig. 2B), with a lowest observed effect concentration (LOEC) of 0.3 µM for both PCBs. The maximum concentration of PCBs used was 10 µM as the solubility of PCBs is limited and higher concentrations lack toxicological relevance. Consequently, it was impossible to determine complete concentration-response curves and to calculate the corresponding EC_{50}s. Although both PCBs have the same LOEC for potentiation of the $GABA_A$ receptor, at higher concentrations (>1 µM) PCB28 is more potent than PCB52 (Fig. 2B). At the highest concentration tested (10 µM), the potentiation of the GABA-induced ion current by PCB28 and PCB52 amounted to 98.2 ± 12.5% (n=7) and 24.5 ± 1.4% (n=8), respectively.

In order to exclude the possibility that NDL-PCBs exert their potentiating effects via another, natively expressed, receptor, sham-injected oocytes (lacking $GABA_A$ receptors) were exposed to the NDL-PCBs that were able to potentiate the $GABA_A$ receptor. None of the tested NDL-PCBs showed any effect (data not shown) in these sham-injected oocytes. Additionally, pharmacologically blocking the GABA-binding site of the $GABA_A$ receptor using gabazine (25 µM), completely abolished the GABA/NDL-PCB effect (not shown), underlining that the NDL-PCB effect is a direct, $GABA_A$ receptor-mediated effect. These findings thus indicate that PCB28 and PCB52 act as partial agonists of the $GABA_A$ receptor *in vitro*.

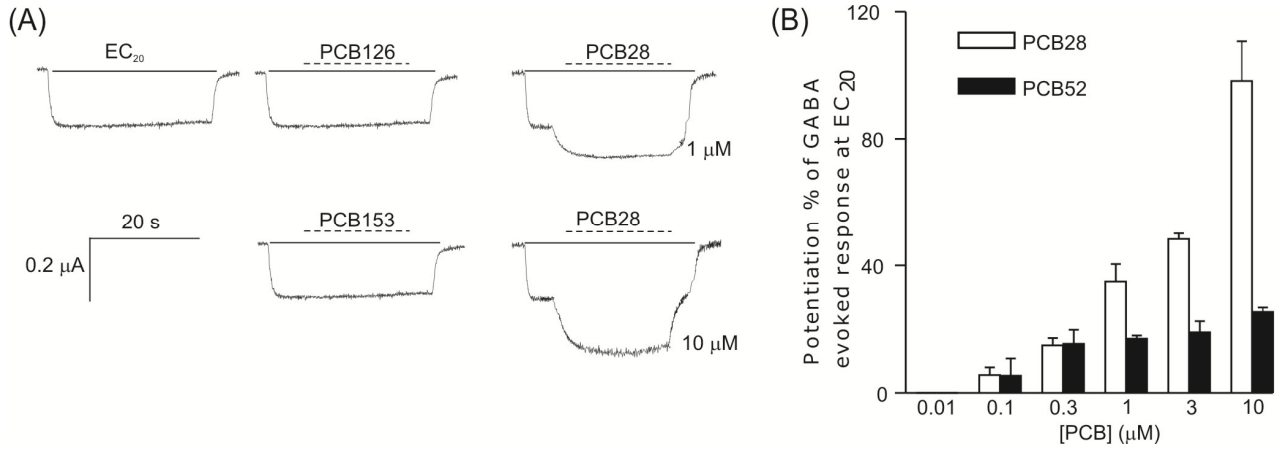

Figure 2. Potentiation by PCB28 and PCB52 of GABA-evoked ion currents under conditions of low receptor occupancy. (A) Example recordings of Cl^- current evoked by GABA at EC_{20} (indicated by the solid line on top of the recording, upper left). During the application of GABA (at EC_{20}), PCBs were co-applied for 20 s (dashed line on top of the recordings). Penta-chlorinated PCBs, like the dioxin-like PCB126 or higher-chlorinated NDL-PCBs, do not affect the GABA-evoked ion current (middle traces). The lower-chlorinated NDL-PCB28 induces a dose-dependent potentiation of the GABA-evoked ion current (right traces). Scale bar applies to all traces. (B) Bar graph demonstrating the concentration-dependent potentiation of GABA-induced responses by PCB28 and PCB52. LOECs, i.e., the lowest PCB concentrations that significantly potentiates the GABA-evoked ion current, amounted to 0.3 μM for both PCBs. Bars represent mean ± S.E.M. (*n*=3-22).

Effects of Binary Mixtures of NDL-PCBs on $GABA_A$ Receptor Activation

PCB28 and PCB52 both potentiate the GABA-evoked ion current. However, human exposure is generally not to individual PCB congeners, but to mixtures of different PCBs, which could result in additive effects. Therefore, oocytes were co-exposed to GABA (at EC_{20}) and an approximately equipotent mixture of PCB28 and PCB52. As shown in Fig. 3A, co-application of GABA (at EC_{20}) with only PCB28 (0.3 μM) or only PCB52 (10 μM) induced a potentiation of the GABA-evoked ion current of 15.0 ± 2.3% and 25.5 ± 1.4%, respectively. Co-application of GABA (at EC_{20}) with a mixture of PCB28 (0.3 μM) and PCB52 (10 μM) evoked a significantly larger potentiation (43.5 ± 7.7%; $p<0.05$) of the GABA-evoked current, demonstrating that under these conditions the potentiating effects of PCB28 and PCB52 are likely additive.

PCB153, which has neurotoxic potency (18) (29), is among the most abundant PCBs found in the environment as well as human and animal samples (8). However, PCB153 (up to 10 μM) was not able to potentiate the GABA-evoked ion current. When oocytes were exposed to GABA (at EC_{20}) with a mixture of PCB28 (0.3 μM) and PCB153 (10 μM), the PCB28-evoked potentiation of the GABA-evoked current was significantly attenuated from 15.0 ± 2.3% to 1.6 ± 0.9% (Fig. 3B; $p<0.05$). These combined results indicate that the composition of the mixture apparently determines

whether additive potentiation (mixture of lower-chlorinated NDL-PCBs) or competitive binding/antagonism (mixture of lower-chlorinated and higher molecular weight NDL-PCBs) occurs.

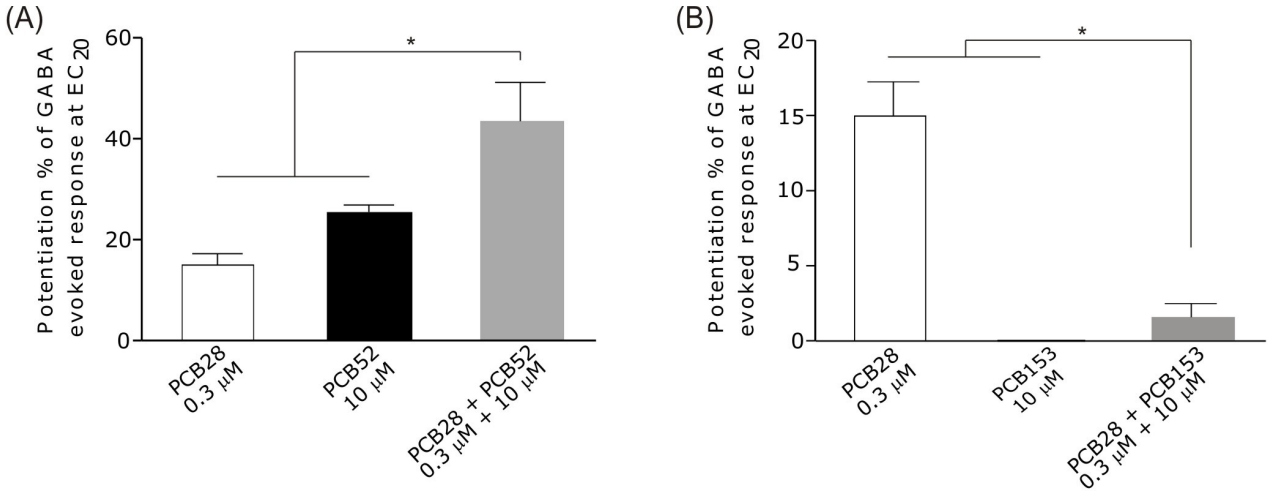

Figure 3. Bar graph demonstrating the potentiation of GABA-induced ion current by PCB28 (0.3 µM), PCB52 (10 µM), PCB153 (10 µM) and PCB mixtures. (A) PCB28 (0.3 µM) and PCB52 (10 µM) both potentiate the GABA-evoked ion current (potentiation amounts to 15.0 ± 2.3% and 25.5 ± 1.4%, respectively). Co-application of GABA at EC_{20} with a mixture of PCB28 and PCB52 potentiated the GABA-evoked ion current, apparently in an additive manner, to 43.5 ± 7.7% (*,p<0.05 compared either of the PCBs alone). (B) PCB153 (10 µM) was not able to potentiate the GABA-evoked ion current. Co-application of GABA at EC_{20} with a mixture of PCB28 (0.3 µM) and PCB153 (10 µM) potentiated the GABA-induced ion current (1.6 ± 0.9%), but the potentiation was strongly attenuated compared to the potentiation of PCB28 alone (15.0 ± 2.3%; *,p<0.05). Data are mean ± S.E.M. (n=7-12).

Discussion

The present results demonstrate a novel mode of action for NDL-PCBs. Lower-chlorinated NDL-PCBs, i.e., PCB28 and PCB52, act as a partial agonist on the human $GABA_A$ receptor, whereas the other tested, higher-molecular weight NDL-PCBs are unable to activate the receptor (Fig. 2A). Potentiation of the GABA-evoked response by PCB28 and PCB52 apparently depends on the level of receptor occupancy as it occurs when GABA is applied at EC_{20}, but not at EC_{80}. Further, the lack of effect of the NDL-PCBs on sham-injected oocytes and the abolishment of the GABA and NDL-PCBs effect by the specific $GABA_A$ receptor antagonist gabazine clearly shows that the NDL-PCB-induced potentiation is a direct, $GABA_A$ receptor-mediated effect. Importantly, in our *in vitro* system, the effects of PCB28 (0.3 µM) and PCB52 (10 µM) on $GABA_A$ receptor potentiation are apparently additive (Fig. 3A), whereas co-application of PCB28 (0.3 µM) and PCB153 (10 µM) attenuated this potentiation (Fig. 3B), suggesting antagonism or competitive binding.

A wide range of toxic properties of PCBs has been earlier described, including adverse effects on the endocrine system, especially effects of PCBs on the AhR and thyroid hormone signaling (for review see (11), (30), (31), (32)). Though several of these endocrine effects are specifically linked to DL-PCBs, a large number of studies reported neurotoxic effects, which are mainly associated with exposure to NDL-PCBs. Exposure, especially developmental exposure, to NDL-PCBs has been reported to induce neurobehavioral and neurological effects, e.g., mild impairment of learning and memory and motor activity (16). Several cellular effects have been described that could underlie these subtle effects, including altered brain neurotransmitter levels due to changes in neurotransmitter synthesis, metabolism and transporters. Moreover, NDL-PCBs have been reported to induce oxidative stress and increase the intracellular Ca^{2+} concentration (for review see (12), (33), (34)).

As PCBs comprise a large number of congeners, previous studies addressed the neurotoxic potential of PCBs according to their chlorination pattern. Inhibition of vesicular neurotransmitter uptake has been shown to be specific for ortho-substituted PCBs, with penta- and hexa-chlorinated PCBs being the most potent (13). Similarly, inhibition of membrane transporter-mediated neurotransmitter uptake is also specifically inhibited by ortho-substituted PCBs, with tetra- and penta-chlorinated PCBs showing the strongest inhibition (14). Further, PCB-induced ryanodine receptor activation (35), Protein Kinase C (PKC) translocation (36), (37), (38) and decrease in cell dopamine content (39) appeared again specific for ortho-substituted PCBs, with penta-chlorinated congeners, especially PCB153, being among the most potent PCBs. Disruption of Ca^{2+} homeostasis has been identified as one of the major neurotoxic effects of NDL-PCBs (40), (41), (42), with penta- and hexa-chlorinated congeners among the most effective PCBs (42).

All these cellular effects are, however, related to presynaptic neurotransmission or cytotoxicity, whereas acute, postsynaptic effects have not been described. Further, our data suggests that the potentiation of the GABA-induce ion currents by NDL-PCBs is exclusive for lower-chlorinated congeners, namely PCB28 and PCB52, whereas higher-chlorinated and dioxin-like PCB126 were unable to potentiate the $GABA_A$ receptor (Fig. 2A). However, additional studies are required to further investigate this suggested Structure Activity Relation (SAR) and the suggested additive effect of lower-chlorinated PCBs on the $GABA_A$ receptor.

Human exposure to NDL-PCBs is not restricted to a single congener and in our *in vitro* system the effects of the binary mixture of PCB28 and PCB52 on the $GABA_A$ receptor activation indicate that these congeners likely interact in an additive way. This property has been described before suggesting that NDL-PCBs can act via the same specific site when perturbing Ca^{2+} homeostasis and causing PKC translocation. (38). However, the present experiments with a binary mixture of PCB28 and PCB153 have shown that the potentiating effect of PCB28 is reduced by the higher-chlorinated congener (Fig. 3B), suggesting antagonism or competitive

binding to the $GABA_A$ receptor. This receptor has several binding sites via which PCBs can exert their effects. PCB153 may exert competitive binding to the $GABA_A$ receptor if it binds to the same binding site as PCB28, though without activating it. Alternatively, PCB28 and PCB153 may also bind to different sites on the same receptor, but with the binding of PCB153 hampering the binding of PCB28, e.g., by shielding the binding site via which PCB28 potentiates the $GABA_A$ response. PCB28 and PCB52 potentiate the GABA-evoked response at EC_{20}, but not at EC80 or in the absence of GABA and this GABA/NDL-PCB effect is abolished by the specific GABA antagonist gabazine. Further, none of the tested PCB congeners was able to inhibit the GABA-evoked ion current. The results thus suggest that lower-chlorinated NDL-PCBs act via one of the potentiating allosteric binding sites, but not via one of the two GABA binding sites or any of the antagonistic binding sites of the $GABA_A$ receptor, e.g., the picrotoxin binding site (21). However more experiments are required to confirm this.

Due to their lower molecular weight, lower-chlorinated PCBs are among the most abundant congeners in indoor air and dust samples in both public and private buildings, due to leakage from PCB containing ceilings and other construction materials. Especially for children and toddlers, inhalation and dust ingestion are major route of exposure to (lower-chlorinated) PCBs (43). Human plasma levels of individual lower-chlorinated PCBs, including PCB28 and PCB52, amount on average to 0.03 nM following exposure via contaminated indoor air (44). The LOECs for PCB28 and PCB52 in our study are in the low μM range (0.3 μM). Despite the nearly four order of magnitude difference between these LOECs and human serum concentration, the reported effects could be relevant if additivity applies, as humans and wildlife are generally exposed to a mixture of NDL-PCBs. Higher-chlorinated PCBs, including PCB153, are among the most abundant congeners found in food and feed, therefore food ingestion is one of the most important routes of exposure to these particular congeners. For the three most abundant NDL-PCBs (PCB138, PCB153 and PCB180), blood levels as high as 50 nM have been reported for people living in a contaminated area in Eastern Slovakia (8), which lowers the margin of safety substantially. However, it should be noted that these higher-chlorinated persistent NDL-PCBs are unable to potentiate the $GABA_A$ receptor. On the contrary, PCB153 can decrease the potentiating effect of PCB28, indicating that the composition of the NDL-PCBs mixture (and the relative concentration of the different congeners) will determine the net effect on the $GABA_A$ receptor.

The six common NDL-PCBs used in this study account for approximately 50% of all the NDL-PCBs present in feed and food. Therefore, the novel mode of action observed in the present experiments should be taken into account for human risk assessment of NDL-PCBs. Additionally, these results also indicate that a thorough exposure characterization for lower-chlorinated NDL-PCBs, and especially their

(toxicological) contribution within a relevant mixture, is one of the prerequisites for human risk assessment for these PCBs.

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Chapter 3

Activation and potentiation of human $GABA_A$ receptors by non-dioxin-like-PCBs depends on chlorination pattern

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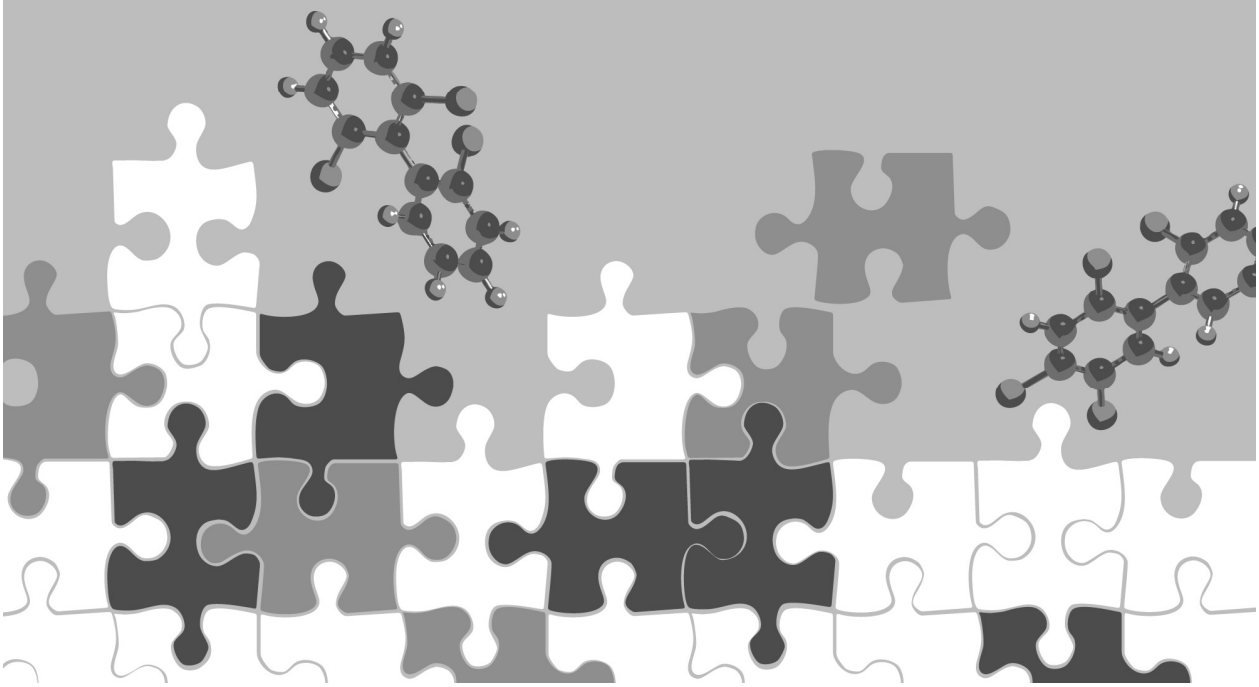

Abstract

The neurotoxic potential of non-dioxin-like PCBs (NDL-PCBs) is characterized by disruption of presynaptic processes, including calcium homeostasis and neurotransmitter transport. Recently, using a limited set of congeners, we demonstrated that PCB28 and PCB52 can potentiate postsynaptic $GABA_A$ receptors. In the present study, effects of twenty NDL-PCBs and two dioxin-like PCBs, selected based on their chemical variation and abundance in the environment, on human $GABA_A$ receptors were investigated. $GABA_A$ receptors were expressed in *Xenopus* oocytes and NDL-PCB effects were determined using the two-electrode voltage-clamp technique.

Results demonstrate that lower-chlorinated PCB19, PCB28, PCB47, PCB51, PCB52, PCB95 and PCB100 act as a partial agonists (at low receptor occupancy), i.e., potentiating the receptor response during co-application with GABA (at EC_{20}). Importantly, PCB19, PCB47, PCB51 and PCB100 can also act as full agonist, i.e., activate the $GABA_A$ receptor in the absence of GABA. Potentiation and activation of the $GABA_A$ receptor is concentration-dependent and limited to NDL-PCBs that have 3-5 chlorine atoms, 1-3 *ortho*-substitutions, an equal number (0-1) of *meta*-substitutions on both phenyl rings and do not have an adjacent *para*- and *meta*-substitution on the same phenyl ring. Activation and potentiation of the $GABA_A$ receptor by PCB47, the most potent congener (LOEC of 10 nM), is attenuated when co-applied with PCB19, PCB28, PCB153 or PCB180, indicative for competitive binding. Considering the importance of GABA-ergic signaling for brain development, motor-coordination and learning and memory, this mode of action can contribute to the previously observed NDL-PCB-induced neurobehavioral and neurodevelopmental effects and should be included in human risk assessment.

Introduction

Polychlorinated biphenyls (PCBs) are a group of persistent organic pollutants (POPs), which have been used in numerous industrial and commercial applications World wide more than 1.5 million tons of PCBs were produced until their production, commercialization and use was largely prohibited in the 1970s (Breivik *et al.*, 2002). Mainly due to improper waste disposal, PCBs still enter the environment and because of their lipophilicity and biopersistence they tend to accumulate in biota (ATSDR, 2000). Food is one of the major routes for human PCB-exposure, though inhalation of (indoor) air and house dust can also provide significant exposure, mainly to lower-chlorinated congeners (Broding *et al.*, 2007, Peper *et al.*, 2005). Regardless the route of exposure, levels of individual PCBs in human blood have been shown to be in the sub- to lower nM range. However, humans are not exposed to a single congener and sum of the levels of the most common PCBs in blood can add up to higher nM range (Petrik *et al.*, 2006, Gabrio *et al.*, 2000).

According to their chemical and toxicological properties, PCBs can be divided into two groups: non-dioxin-like PCBs (NDL-PCBs) and dioxin-like PCBs (DL-PCBs). Contrary to DL-PCBs, NDL-PCBs have one or more chlorines in the *ortho*-positions, resulting in non-planar structures. Consequently, NDL-PCBs have little or no affinity to the AhR and display a different toxicological profile. Several epidemiological studies have indicated that perinatal exposure to NDL-PCBs can result in neurodevelopmental and neurobehavioral effects in children (Faroon *et al.*, 2001, Winneke *et al.*, 1998). Moreover, *in vivo* studies have shown that animals dosed with NDL-PCBs displayed neurobehavioral effects, including changes in motor activity, learning, memory and attention (Eriksson *et al.*, 2006, Fischer *et al.*, 2008, Holene *et al.*, 1998, Boix *et al.*, 2010). Further research suggested the association of these effects with the disruption of thyroid hormone metabolism (Knerr and Schrenk, 2006) and with alterations in excitatory glutamergic and dopaminergic neurotransmitter systems as well as with the inhibitory GABA-ergic system (Chu *et al.*, 1996, Kim *et al.*, 2009).

In vitro studies indentified regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$), which is essential for neural activity and cell viability, as one of the critical parameters affected by NDL-PCBs (Kim *et al.*, 2009, Tilson and Kodavanti, 1998, Kodavanti *et al.*, 2001, Shafer *et al.*, 1996). Additional *in vitro* studies revealed that NDL-PCBs can also affect other presynaptic processes essential for proper neurotransmission, such as inhibition of the uptake of dopamine, serotonin, glutamate and GABA in synaptosomes and synaptic vesicles isolated from rat brain (Mariussen *et al.*, 2001, Mariussen and Fonnum, 2001).

With respect to postsynaptic processes, acute, direct effects of NDL-PCBs on postsynaptic $GABA_A$ receptors have been described only recently (Antunes Fernandes *et al.,* 2010). $GABA_A$ is a pentameric receptor in which $\alpha_1\beta_2\gamma_2$ is the most common

subunit composition in central nervous system (CNS) (for review see (Mohler 2007)). GABA binds to the receptor via its two specific binding sites (between the α and β subunits), resulting in an opening of the receptor and a subsequent Cl^- current across the membrane. There are, however, several other binding sites that are capable of modulating $GABA_A$ receptor function and depending on the binding site involved, the GABA-induced Cl^- current can be potentiated or inhibited. Modulation of the $GABA_A$ receptor is of particular relevance as GABA is the main inhibitory neurotransmitter in the adult mammalian CNS and provides the main inhibitory feedback for learning and memory as well as motor activity (for review see (Mohler, 2007)).

Based on six abundant NDL-PCBs, it was shown that lower-chlorinated congeners are more effective in potentiating the $GABA_A$ receptor than higher-chlorinated NDL-PCBs (Antunes Fernandes *et al.*, 2010). However, only a limited set of NDL-congeners was studied, hampering identification of a structure-activity relation (SAR) for NDL-PCBs. Further, studying six congeners, it was unclear whether NDL-PCBs are also able to modulate the $GABA_A$ receptor in the absence of GABA, i.e, act as full agonist on the human $GABA_A$ receptor.

Based on their physical-chemical properties and abundance in food and human samples, Stenberg and Andersson have described a selection of 20 NDL-PCBs (Stenberg and Andersson, 2008). In the present study this set of NDL-PCBs and two DL-PCBs was used to measure acute effects of NDL-PCBs on $GABA_A$ receptor functioning and to elucidate a SAR. Further, as human exposure is not restricted to single congeners, we also investigated the effects of binary mixtures of NDL-PCBs on $GABA_A$ receptor function.

Experimental Section

Animals

All experiments were in accordance with Dutch law and approved by the Utrecht University Ethical Committee for Animal Experiments. Adult female specimen of *Xenopus laevis* frogs (provided by Dr. Wim Scheenen, Radboud University, Nijmegen, The Netherlands) were kept in copper-free tapwater (pH 6.5, 23^oC) in standard aquaria (0.5 × 0.4 × 1 m; 1-15 per aquarium) with a 12 h light/dark cycle. The animals were fed earthworms (Hagens, Nijkerkerveen, The Netherlands) three times a week.

Chemicals

GABA, gabazine, neomycin solution (10 mg neomycin/ml in 0.9% NaCl), collagenase type I, NaCl and 3-aminobenzoic acid ethyl ester (MS-222) were obtained from Sigma Chemical (St. Louis, MO, USA). $CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution),

$MgSO_4$, $NaHCO_3$, NaOH, $Ca(NO_3)_2$, KCl, and HEPES were purchased from Merck (Darmstadt, Germany). cDNA of human $GABA_A$ subunits was synthesized and provided by Paul J. Whiting (Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Harlow, Essex, UK).

The 22 PCBs used in this study were PCB19, PCB28, PCB47, PCB51, PCB52, PCB53, PCB74, PCB77, PCB95, PCB100, PCB101, PCB104, PCB118, PCB122, PCB126, PCB128, PCB136, PCB138, PCB153, PCB170, PCB180 and PCB190 (see Table 1 of supplementary data for full names and number of *ortho*-chlorine substitution of each congener). PCBs were purchased from Neosync Inc., USA and possible impurities, e.g., polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/Fs) and DL-PCBs, were removed by applying the PCBs dissolved in *n*-hexane on an active carbon column and collecting them after elution with n-hexane as described by Danielsson *et al* (2008). The highly purified PCBs were dissolved in purity-checked dimethyl sulfoxide (DMSO). PCB stock solutions of 25 mM were further diluted to obtain a final experimental concentration ranging from 1 nM to 10 µM. DMSO at concentrations up to 0.5% (v/v) had no effect on the $GABA_A$ receptor mediated Cl^- currents. In the present experiments, the final concentration of DMSO in PCB containing saline was always kept below 0.1% (v/v).

Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ Receptors in Xenopus laevis Oocytes

Oocytes from *Xenopus laevis*, injected with foreign cDNA of the receptor of choice, are a commonly used tool in studying direct effects plasma membrane receptors (for review see (Sigel and Minier, 2005)). Oocyte preparation and injection was as described previously (Antunes Fernandes *et al.*, 2010). Briefly, female *Xenopus laevis* were anaesthetized by submersion in 0.1% MS-222 and ovarian lobes were surgically removed. Oocytes were treated with collagenase type I (1.5 mg/ml Ca^{2+} free Barth's solution) for 90 min at room temperature before manual defolliculation. cDNA coding for the human α_1, β_2, and γ_{2L} subunits of human $GABA_A$ receptors, dissolved in distilled water at a 1:1:1 molar ratio, was injected into the nuclei of stage V or VI oocytes using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA, USA). The injected volume was 23 nl/oocyte (~1 ng of each subunit). Sham-injected oocytes were injected only with 23 nl of distilled-water, i.e., without cDNA. Following injection of the cDNA, or only demi-water, oocytes were incubated at 21°C in modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 10 µg/ml neomycin (pH 7.6 with NaOH). Experiments were performed on oocytes after 2-5 days of incubation, i.e., when receptor expression is maximal. Each experiment was repeated with oocytes obtained from at least two different animals.

Electrophysiological Recording

Ion currents associated with $GABA_A$ receptor activity were measured with the two-electrode voltage-clamp technique using a Gene Clamp 500B amplifier (Axon Instruments) with high-voltage output stage as described previously (Antunes Fernandes *et al.*, 2010). Recording microelectrodes (0.5-2.5 MΩ) were filled with 3 M KCl. Oocytes, placed in a Teflon recording tube, were voltage clamped at -60 mV and continuously superfused (~30 ml/min) with saline solution, containing (in mM): 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH). Aliquots of freshly thawed stock solutions of GABA (1 M) in demi-water and of the different PCBs in DMSO were added to the saline immediately before the experiments. Oocytes were exposed to compounds by switching the perfusate from saline to PCB and/or GABA-containing saline using a servomotor-operated valve. The maximum PCB concentration tested was 10 µM as the solubility of PCBs is limited and higher concentrations lack toxicological relevance. Consequently, it was not possible to establish complete concentration-response curves and to calculate the corresponding EC_{50}s for all PCBs. For specific experiments, oocytes were exposed to a binary mixture of PCBs (1 µM PCB47 + 10 µM PCB19; 1 µM PCB47 + 10 µM PCB28 or 1 µM PCB47 + 10 µM PCB180) or to a mixture of GABA (at EC_{20} concentration) and PCBs (0.01 µM PCB47 + 10 µM PCB153). In order to minimize absorption of PCBs to the perfusion system, glass reservoirs and Teflon tubes (PTFE; 4x6mm, Rubber, Hilversum, The Netherlands) were used.

Oocytes were repeatedly exposed to different GABA- and/or PCB-containing solutions. To correct for desensitization or run-up, oocytes were superfused with GABA-containing saline to evoke a control response (EC_{max} or EC_{20}) before and after each PCB exposure, so each oocyte could serve as its own control. Further, between each application a washout period of 2-5 min was introduced to allow receptors to recover from possible desensitization. The lipophilic nature of the PCBs apparently did not affect the observed rapidly reversible potentiation of the GABA-evoked response, as repeated applications of PCBs did not change the observed effects (data not shown). Membrane currents were low-pass filtered (8-pole Bessel; 3 dB at 0.3 kHz), digitized (12 bits; 1024 samples/record), and stored on disk for computer analysis.

Data Analysis and Statistics

Peak amplitudes of GABA-induced or PCB-induced ion currents were measured and normalized to the maximum amplitude of GABA-induced responses (1 mM) to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitude over time. The percentage of the PCB receptor activation was calculated as a quotient of the maximum amplitude of PCB and GABA

(at 1 mM). The percentage of PCB-induced potentiation of the GABA-induced ion current was calculated from the quotient of the maximum amplitude of the GABA-PCB co-application response and the maximum amplitude of the control response (GABA at EC_{20} concentration). As such, the effects of the PCB exposure are always expressed as a % of control, with each oocyte serving as its own control, corrected for changes over time. Data are expressed as mean ± SEM of *n* oocytes. Statistical differences ($p<0.05$) were calculated using paired and unpaired two-tailed Student's *t*-test where appropriate.

Results

Effects of NDL-PCBs on $GABA_A$ Receptor Activation

Oocytes, voltage clamped at -60 mV, were exposed to saline containing 0.3 µM to 3 mM GABA and GABA-induced inward Cl^- currents were normalized to the maximum GABA-induced current (1 mM) to obtain a GABA concentration-response curve (data not shown). This curve was used to determine the EC_{20} and EC_{80}, i.e., concentrations producing 20% and 80% of maximal response, which amounted to 22 and 193 µM, respectively (*n*=10; Antunes Fernandes *et al.*, 2010).

First, twenty NDL-PCBs and two DL-PCBs (supplementary Table 1) were screened for possible agonistic effects on the human $GABA_A$ receptor by exposing oocytes to PCB-containing (1 or 10 µM) saline. It was previously shown that PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180 were unable to activate the $GABA_A$ receptor (Antunes Fernandes *et al.*, 2010). In the present study, the majority of the tested PCBs were also unable to act as full agonist at the $GABA_A$ receptor (see Table 2). However, PCB19, PCB47, PCB51 and PCB100 were able to activate the $GABA_A$ receptor. Subsequently, these four congeners were tested at lower concentrations (30 nM - 10 µM) to determine the LOEC (Lowest Observed Effect Concentration) and, if possible, the EC_{50} for activation of the $GABA_A$ receptor (Figure 1, Table 1 and supplementary Figure 1). At 1 µM, PCB47 clearly induced $GABA_A$ receptor activation, whereas at 0.1 µM and 0.3 µM this induction was very modest and not concentration-dependent. Still, PCB47 was the most potent tested congener, with a LOEC for $GABA_A$ receptor activation of 0.1 µM (*n*=3), an EC_{50} of 0.67 µM and maximum activation of the $GABA_A$ receptor amounting to 42.5 ± 4.5% at 3 µM (*n*=3). However, at 10 µM activation was attenuated to 25.0 ± 11.8% (not significant compared to 3 µM; *n*=4). PCB51 showed comparable but less potent effects (LOEC: 0.3 µM; EC_{50}: 1.1 µM) with a maximum activation of only 6.9 ± 1.6% at 3 µM (*n*=5). Again, at 10 µM receptor activation decreased (0.07 ± 0.04%, $p<0.05$; *n*=3). PCB19 and PCB100 had the highest LOEC, amounting to 3 µM (*n*=4) and 10 µM (*n*=8), respectively. At 10 µM both PCBs showed a comparable maximum activation of the $GABA_A$ receptor; 18.3 ± 7.0% (PCB19, *n*=4) and 12.1 ± 3.6% (PCB100, *n*=8).

These results thus demonstrate that some lower-chlorinated NDL-PCBs can act as full agonist on the human $GABA_A$ receptor and that activation of the $GABA_A$ receptor is at least partly dependent on the chlorination pattern of the congener.

Table 1. Summary of the effects of NDL-PCBs on human $GABA_A$ receptor function. Effects can be classified as partial (left) and full (right) agonistic, i.e., potentiation and activation of the receptor, respectively. Values for maximum activation are presented as mean ± SEM (*n*=3-19). N.D Not determined, indicating that EC_{50} could not be determined as the maximum potentiating effect was not yet reached at the highest concentration tested (10 µM). [(a)] Results described previously (Antunes Fernandes *et al.*, 2010).

Congener	Partial agonist			Full agonist		
	LOEC	**EC_{50}**	**Maximum Potentiation**	**LOEC**	**EC_{50}**	**Maximum Activation**
PCB19	0.3 µM	2.4 µM	119.5 ± 15.6% (10 µM)	3 µM	N.D.	18.3 ± 7.0% (10 µM)
PCB28[(a)]	0.3 µM	N.D.	98.2 ± 12.5% (10 µM)	-	-	-
PCB47	0.01 µM	0.17 µM	223.4 ± 34.2% (3 µM)	0.1µM	0.67 µM	42.5 ± 4.5% (3 µM)
PCB52[(a)]	0.3 µM	N.D.	24.5 ± 1.4% (10 µM)	-	-	-
PCB51	0.03 µM	1.03 µM	168.0±39.6% (3 µM)	0.3 µM	1.1 µM	6.9 ± 1.6% (3 µM)
PCB95	0.3 µM	0.34 µM	20.0 ± 3.5% (1 µM)	-	-	-
PCB100	0.03 µM	0.15 µM	122.1 ± 17.6% (3 µM)	10 µM	N.D.	12.1 ± 3.6% (10 µM)

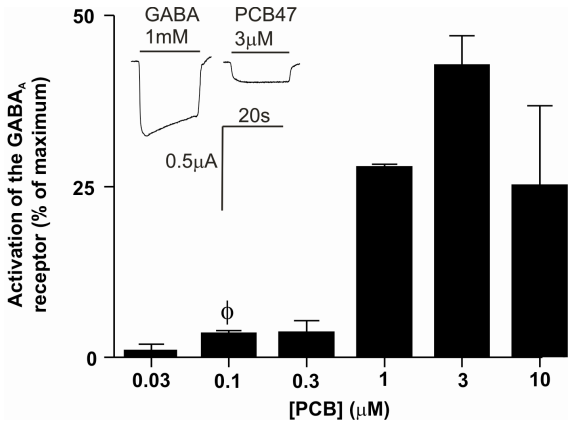

Figure 1. **GABA$_A$ receptor activation by PCB47.** Activation of the human GABA$_A$ receptor is presented as % of the maximum GABA-evoked response (1 mM). Inset shows example recordings of the maximum Cl^- current evoked by GABA (1 mM; left) and by PCB47 (3 μM; right), as indicated by the lines on top of the recordings. The bar graph demonstrates the concentration-dependent activation of the GABA$_A$ receptor by PCB47. Bars represent mean ± SEM. (*n*=3-19). ϕ LOEC, *p*<0.05) See Table 1 and supplementary material for data on activating effects of PCB19, PCB51 and PCB100.

Effects of NDL-PCBs on GABA$_A$ Receptor Potentiation at Low Receptor Occupancy

As previously reported (Antunes Fernandes *et al.*, 2010), at low receptor occupancy (EC_{20}) the lower-chlorinated PCB28 and PCB52 are able to potentiate the GABA-induced current in a concentration-dependent manner. However, this potentiation effect is not seen at high receptor occupancy (EC_{80}). In the present study, none of the twenty NDL-PCBs and two DL-PCBs tested (1 μM or 10 μM) showed any agonistic or antagonistic properties when co-applied with GABA at EC_{80} concentration (data not shown). However, when co-applied with GABA at EC_{20}, PCB19, PCB47, PCB51, PCB95 and PCB100 were able to potentiate the GABA-evoked current (Figure 2, Table 1 and supplementary Figure 2.). These PCBs were further tested at a concentration ranging from 1 nM to 10 μM.

Again, PCB47 was the most potent NDL-PCB with a LOEC for potentiation of the GABA$_A$ receptor of 0.01 μM (*n*=9) and an EC_{50} of 0.17 μM. Maximum potentiation was reached at 3 μM and amounted to 223.4 ± 34.2% (*n*=5). Although not statistically significant, the potentiating effect attenuated at 10 μM (198.3 ± 13.2 %; *n*=3). Potentiation of the GABA$_A$ receptor by PCB51 and PCB100 was comparable, though less potent than PCB47. Both PCBs had a LOEC of 0.03 μM and a maximum potentiation of the GABA$_A$ receptor of approximately 120-160% at 3 μM. However, the EC_{50} of PCB51 (1.03 μM) was approximately one order of magnitude higher that the EC_{50} of PCB100 (0.15 μM). Comparable with PCB28 and PCB52 (Antunes Fernandes *et al.*, 2010), PCB19 and PCB95 have a LOEC for potentiation of the GABA$_A$ receptor of 0.3 μM. However, at higher concentrations (>1 μM), PCB19 and PCB28

were more effective in potentiating the $GABA_A$ receptor than PCB52 and PCB95 (see Table 1 for details). These results thus demonstrate that several lower-chlorinated NDL-PCBs can act as partial agonist on the human $GABA_A$ receptor when co-applied with GABA at EC_{20} and that this action depends at least on the chlorination pattern of the congener.

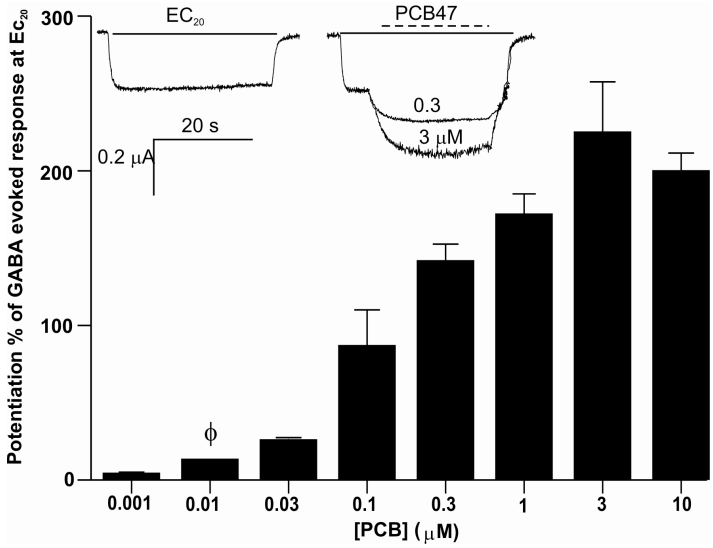

Figure 2. $GABA_A$ receptor potentiation by PCB47. Potentiation of the human $GABA_A$ receptor is presented as % of the GABA-evoked response at EC_{20} (22 μM). Inset shows example recordings of the Cl^- current evoked by GABA (EC_{20}; left) and its potentiation by PCB47 (0.3 and 3 μM; right), as indicated by the lines on top of the recordings). The bar graph demonstrates the concentration-dependent potentiation of GABA-induced response, at low receptor occupancy (EC_{20}) by PCB47. Bars represent mean ± SEM. (*n*=3-19). ϕ LOEC, $p<0.05$). See Table 1 and supportive material for data on the potentiating effects of PCB19, PCB28, PCB51, PCB52, PCB95 and PCB100.

Effects of Binary NDL-PCB Mixtures on $GABA_A$ Receptor Activity

Humans are generally not exposed to one single PCB congener. Moreover, it was previously suggested that higher-chlorinated NDL-PCBs (PCB153) can competitively bind to the $GABA_A$ receptor, thereby attenuating the potentiating effects of PCB28 and PCB52 (Antunes Fernandes *et al.*, 2010). In order to further investigate if the composition of a PCB mixture determines its net effect on $GABA_A$ receptor activation or potentiation, several binary mixtures of NDL-PCBs have been tested.

In the present study, PCB47 is the most potent congener with respect to potentiation of the $GABA_A$ receptor at low receptor occupancy (EC_{20}). Conversely, PCB153 (up to 10 μM) was not able to potentiate the GABA-evoked current. When oocytes were co-exposed to GABA (at EC_{20}) and a binary mixture of PCB47 (0.01 μM) and PCB153 (10 μM), the degree of receptor potentiation evoked by PCB47 alone

decreased from 11.7 ± 1.4 to 3.5 ± 1.1% (p<0.05; Fig. 3A). In line with previous research (Antunes Fernandes *et al.*, 2010), this suggests that the higher-chlorinated PCB153 competitively binds to the $GABA_A$ receptor, without being able to potentiate it.

To investigate the possibility of competitive binding by other NDL-PCBs with respect to $GABA_A$ receptor activation (instead of potentiation), oocytes were first exposed to 1 µM of the full agonist PCB47. The PCB47-induced ion current was set at 100% and oocytes were subsequently exposed to binary mixtures of PCB47 (1 µM) with another but less potent full agonist (PCB19; 10 µM), with a partial agonist (PCB28; 10 µM) or with a higher-chlorinated NDL-PCB (PCB180; 10 µM). All binary mixtures reduced the PCB47-evoked ion current to respectively 73.3 ± 2.4% (n=3; p<0.01), 40.4 ± 1.7% (n=3; p<0.001) and 17.6 ± 0.7% (n=3; p<0.001; Fig 3B). In a comparable set of experiments, oocytes were first exposed to 10 µM of the full (but weak) agonist PCB19 (PCB19-evoked current set at 100%) and subsequently to a binary mixture of PCB19 (10 µM) and PCB47 (1 µM). In this case, the binary mixture increased the inward current evoked by PCB19 to 883.6 ± 26.7% (n=3; p<0.01; Fig 3C). These data thus suggest competitive binding to the $GABA_A$ receptor and that the presence of less potent or inactive PCBs in the mixture attenuates the effect of more potent PCBs.

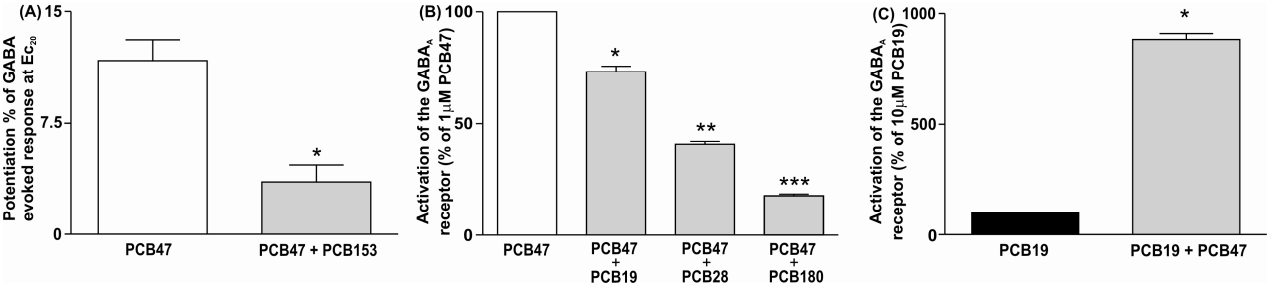

Figure 3. $GABA_A$ receptor potentiation and activation by binary NDL-PCB mixtures. (A) Potentiation of GABA-induced ion current (at EC_{20}) by PCB47 (0.01 µM) is reduced when co-applied as binary mixture with PCB153 (10 µM) (*, p<0.01). **(B)** Activation of the $GABA_A$ receptor by PCB47 (1 µM) was set as 100% to facilitate comparison with the effect of PCB47 in subsequent co-application with PCB19 (10 µM), PCB28 (10 µM) or PCB180 (10 µM) (*, p<0.01, **, p<0.001, ***, p<0.0001). **(C)** Activation of the $GABA_A$ receptor by PCB19 (10 µM) was set as 100% to facilitate comparison with the effect of PCB19 in subsequent co-application with PCB47 (1 µM) (*, p<0.001). Bars represent mean ± SEM. (n=3-4).

Discussion

Previously, Antunes Fernandes *et al.,* (Antunes Fernandes *et al.*, 2010) identified potentiation of the $GABA_A$ receptor by the lower-chlorinated NDL-PCBs 28 and 52 as a novel mode of action. The present study shows that also PCB19, PCB47, PCB51, PCB95 and PCB100 have comparable effects (Figure 2 and supplementary

Figure 2). This potentiating effect is observed only when receptor occupancy is low, i.e., when co-applied with GABA at EC_{20}. Interestingly, PCB19, PCB47, PCB51 and PCB100 are also able to concentration-dependently activate the $GABA_A$ receptor, i.e, producing an inward Cl^- current in the absence of the endogenous agonist GABA (Figure 1 and supplementary Figure 1). Some NDL-PCBs thus mimic the effect of natural agonist GABA, possibly resulting in an indirect interaction of other modulators of the $GABA_A$ receptor. This may be translated into an exacerbation of the effect of partial agonists of the $GABA_A$ receptor, such as neurosteroids or ethanol.

The observed effects on the $GABA_A$ receptor are specific and not due to activation of another, natively-expressed receptor as sham-injected oocytes exposed to the NDL-PCBs did not show any effect (data not shown). Moreover, the onset and washout of the PCB effect is very rapid (both within seconds) and the PCB-induced activation/potentiation of the GABA-response remains stable over time as well as over repeated applications. It is thus unlikely that the observed effects on the $GABA_A$-receptor are due to indirect PCB-induced effects, e.g., on intracellular calcium levels. Experiments with the partial agonist PCB28 and gabazine, which blocks the GABA-binding site, have shown that the potentiating effects of PCBs at least involve the GABA binding site (Antunes Fernandes *et al.,* 2010), likely in combination with a positive allosteric site, e.g., the benzodiazepine binding site. However, the lack of truly specific agonists or antagonists for the different positive allosteric sites precludes more detailed assessment of the binding sites involved.

NDL-PCBs can thus act as (partial) agonist on the human $GABA_A$ receptor. However, as full agonist, PCB47 and PCB51 show a clear reduction in the activation of the $GABA_A$ receptor at higher concentrations (10 µM). Similarly, as partial agonist, i.e., co-applied with GABA at EC_{20}, PCB47, PCB51 and PCB100, show a clear attenuation of receptor potentiation at 10 µM (Table 1, Figure 2 and supplementary Figure 2.). Whether PCB19, PCB28 and PCB95 show the same tendency is not clear because the highest tested concentration (10 µM) was not enough to evoke a complete concentration response curve and higher concentrations lack toxicological relevance and are precluded due to the limited solubility of PCBs. Attenuation of receptor responses is observed for several $GABA_A$ receptor agonists at higher concentrations and is possibly due to rapid receptor desensitization (Akk and Steinbach, 2000, Muroi *et al.*, 2009, Pistis *et al.*, 1997). Alternatively, it can be suggested that these NDL-PCBs bind to an additional (low-affinity) inhibitory binding site of the $GABA_A$ receptor or exert their inhibitory effect indirectly, e.g., due to partitioning in the membrane (Akk *et al.,* 2009*,* Hosie *et al.,* 2003).

LOECs and EC_{50}s for activation and potentiation of the $GABA_A$ receptor differ by more than one order of magnitude. Additionally, the maximum level of activation or potentiation differs considerably between the different NDL-PCBs (Table 1), making it difficult to establish a general rank-order potency. It is however evident that in this study PCB47 is the most potent NDL-PCB, both as partial and as full

agonist. Human exposure to PCBs is generally not exclusive to a single congener, but to a mixture of PCBs. Our previous results suggested that the potentiating effects of PCB28 and PCB52 are apparently additive. Conversely, co-application of PCB153 and PCB28 attenuated the potentiation of the $GABA_A$ receptor by PCB28, suggesting competitive binding (Antunes Fernandes *et al.*, 2010). In line with this, the present results demonstrate that the potentiating effect of PCB47 is decreased by co-application with PCB153. Moreover, activation of the receptor by the most potent congener, PCB47, is decreased by co-application with the less potent full agonist PCB19, partial agonist PCB28 or high-chlorinated non-active PCB180. Further, activation of $GABA_A$ receptor by PCB19 is enhanced by subsequent application of PCB47. This supports the idea that competitive binding is involved, as observed previously for disruption of thyroid hormone receptor, estrogen receptor and AhR binding (Ucan-Marin *et al.*, 2010, Gutleb *et al.*, 2010, Chauhan *et al.*, 2000, Petrulis and Bunce, 2000).

Several epidemiological and *in vivo* studies have described neurotoxic effects of NDL-PCBs following perinatal exposure. These effects could be at least partly explained by *in vitro* findings where NDL-PCBs were shown to alter neurotransmitter levels due to changes in neurotransmitter synthesis, metabolism and transporters. Further, exposure to NDL-PCBs has been reported to induce oxidative stress and disrupt Ca^{2+} homeostasis (for review see *(*Pessah *et al,* 2010*,* Kodavanti *et al,* 2006*))*. As PCBs comprise a large number of congeners, previous studies have tried to classify their neurotoxic potential according to their chlorination pattern. It was shown that especially *ortho*-substituted congeners were able to inhibit vesicular and membrane neurotransmitter uptake (Mariussen *et al.*, 2001, Mariussen and Fonnum, 2001). Disruption of Ca^{2+} homeostasis has been ascribed primarily to activation of intracellular ryanodine receptors, which appears to be specific for *para*-substituted NDL-PCBs (Kodavanti *et al.*, 1996, Pessah *et al.*, 2010). For activation or potentiation of $GABA_A$ receptors *para*-substitutions are not a prerequisite. Importantly, the observed effects are apparently dependent on the chlorination pattern of the PCBs, as DL-PCB77 and DL-PCB126 as well as NDL-PCBs with more than five chlorine atoms were unable to activate or potentiate the $GABA_A$ receptor. Moreover, the number of *ortho*-substitutions, which to a large extent determines the planarity of the molecule, should be limited to 1-3. For example, the tri-*ortho*-substituted NDL-PCB 51 and 100 are full agonists, whereas the structurally comparable but inactive PCB 104 has four *ortho*-substitutions. Additionally, NDL-PCBs that have both a *para*- and a *meta*-substitution on the same phenyl ring are inactive. For example, PCB 74, which has adjacent *para*- and *meta*-substitutions on one phenyl ring, is inactive, whereas PCB 28, which is structurally similar except for the *meta*-substitution, acts as $GABA_A$ receptor agonist. Similarly, PCB 52 (partial agonist) and PCB 101 (inactive) are structurally similar, except for an adjacent additional *meta*-substitution in PCB101. Finally, based on the current data set it appears that both phenyl rings should have

an equal number of *meta*-substitutions. For example, PCBs 52 and 95 both have a single meta-substitution on each phenyl ring and act as partial agonist, whereas PCB53, which has a *meta*-substitution on only one ring, is inactive. Based on the present selection of 22 PCBs, it is suggested that to be (partial) $GABA_A$ receptor agonist NDL-PCBs should 1) have no more than five chlorine atoms, 2) have 1-3 *ortho*-chlorinated positions, 3) not have an adjacent *para*- and *meta*-substitution on the same phenyl ring and 4) have an equal number (0-1) of *meta*-substitutions on both phenyl rings. Though this proposed SAR is based on 22 PCBs, future experiments will have to prove if this SAR is conclusive. Nonetheless, considering their LOECs and EC_{50}s, the most active NDL-PCBs (i.e. PCB47 and PCB51) appear to be di- and tri-*ortho* substituted, with one or two *para*-substitutions but no *meta*-substitutions.

Low-molecular-weight NDL-PCBs are among the most abundant congeners in contaminated indoor air and dust samples in both public and private buildings (Harrad *et al.*, 2010). Human plasma levels of individual lower-chlorinated PCBs, including PCB28 and PCB47, amount on average to 0.03 nM and 0.02 nM, respectively, following exposure via contaminated indoor air. LOECs for activation of the $GABA_A$ receptor derived from this study are between 0.1 µM and 10 µM. Though LOECs for receptor potentiation are one order of magnitude lower (0.01 µM for PCB47), these concentrations are well above the levels of individual lower chlorinated NDL-PCBs present in human plasma (in the low nM range). While at low concentrations additivity might occur, it should also be noted that the effect of the most potent PCB may be attenuated by the presence of less potent or inactive PCB due to competitive binding (Figure 3).

Nonetheless, the effect concentrations reported here, as low as 10 nM for $GABA_A$ receptor potentiation by PCB47, are in the same range or even well below the effect concentration previously reported for presynaptic adverse effects, including disruption of calcium homeostasis and neurotransmitter transport (Fonnum *et al.*, 2006). Moreover, GABA is essential in early brain development (for review see (Ben-Ari *et al.*, 2007, Owens and Kriegstein, 2002)) and a key player in learning and memory as well as motor activity (for review see (Mohler, 2007)). Consequently, the present data, demonstrating disruption of $GABA_A$ receptor-mediated signaling, likely underlie at least part of the described neurobehavioral and neurodevelopmental effects following NDL-PCB exposure. As such, these findings justify a thorough exposure-characterization and extensive human risk assessment for lower-chlorinated NDL-PCBs.

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Supplementary Data

Table 1 – overview of the congeners used in the present study. Inclusion criteria are described in Stenberg and Andersson (2008).

Congener	Number of *ortho*-chlorine substitution	Full name
PCB19	Tri-*ortho*	2,2',6-Trichlorobiphenyl
PCB28	Mono-*ortho*	(2,4,4'-Trichlorobiphenyl
PCB47	Di-*ortho*	2,2',4,4'-Tetrachlorobiphenyl
PCB51	Tri-*ortho*	2,2',4,6'-Tetrachlorobiphenyl
PCB52	Di-*ortho*	2,2',5,5'-Tetrachlorobiphenyl
PCB53	Tri-*ortho*	2,2',5,6'-Tetrachlorobiphenyl
PCB74	Mono-*ortho*	2,4,4',5-Tetrachlorobiphenyl
PCB95	Tri-*ortho*	2,2',3,5',6-Pentachlorobiphenyl
PCB100	Tri-*ortho*	2,2',4,4',6-Pentachlorobiphenyl
PCB101	Di-*ortho*	2,2',4,5,5'-Pentachlorobiphenyl
PCB104	Tetra-*ortho*	2,2',4,6,6'-Pentachlorobiphenyl
PCB118	Mono-*ortho*	2,3',4,4',5-Pentachlorobiphenyl
PCB122	Mono-*ortho*	2',3,3',4,5,-Pentachlorobiphenyl
PCB128	Di-*ortho*	2,2',3,3',4,4'-Hexachlorobiphenyl
PCB136	Tetra-*ortho*	2,2',3,3',6,6'-Hexachlorobiphenyl
PCB138	Di-*ortho*	2,2',3,4,4',5'-Hexachlorobiphenyl
PCB153	Di-*ortho*	2,2',4,4',5,5'-Hexachlorobiphenyl
PCB170	Di-*ortho*	2,2',3,3',4,4',5-Heptachlorobiphenyl
PCB180	Di-*ortho*	2,2',3,4,4',5,5'-Heptachlorobiphenyl
PCB190	Di-*ortho*	2,3,3',4,4',5,6,-Heptachlorobiphenyl
PCB77	Non-*ortho*	3,3',4,4'-Tetrachlorobiphenyl
PCB126	Non-*ortho*	3,3',4,4',5-Pentachlorobiphenyl

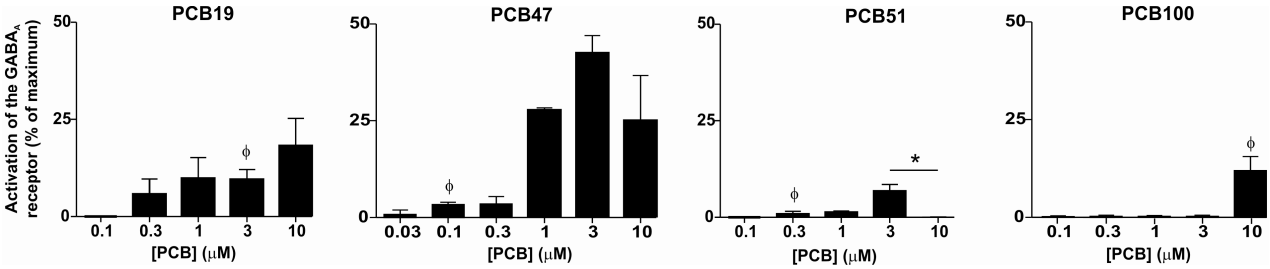

Figure 1. **GABA$_A$ receptor activation by PCB19, PCB47, PCB51 and PCB100.** Activation of the human GABA$_A$ receptor is presented as % of the maximum GABA-evoked response (1 mM). Bars represent mean ± S.E.M. (*n*=3-8); * *p*<0.05; ϕ Lowest Observed Concentration Effect (LOEC); **p*<0.05).

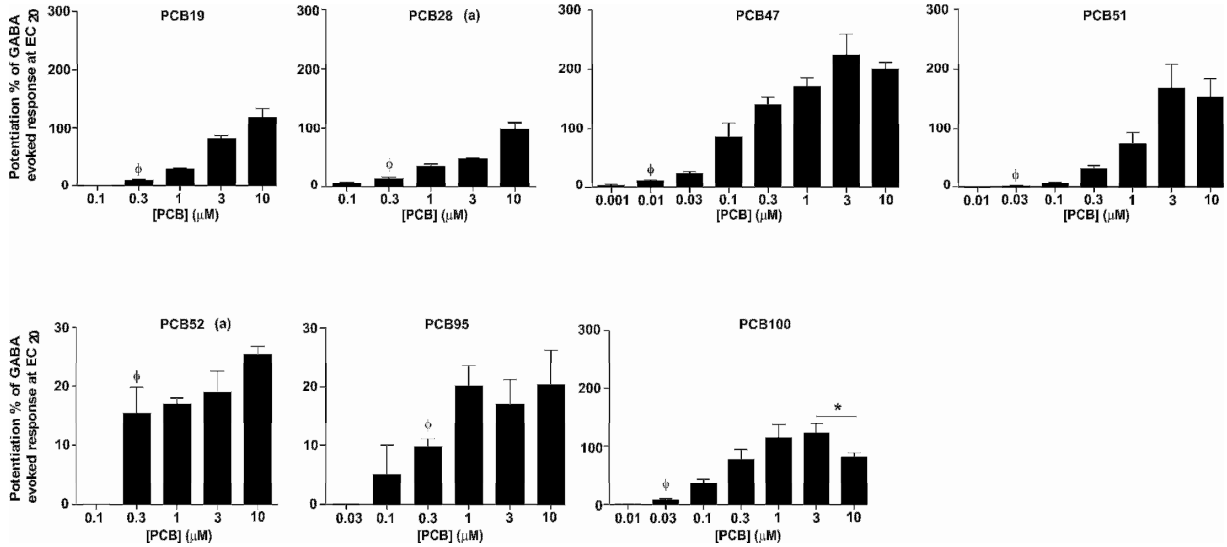

Figure 2. GABA$_A$ receptor potentiation by PCB19, PCB47, PCB51, PCB95 and PCB100. Potentiation of the human GABA$_A$ receptor is presented as % of the GABA-evoked response at EC_{20}. Bars represent mean ± S.E.M. (*n*=3-19); * *p*<0.05; ϕ Lowest observed concentration effect (LOEC); **p*<0.05). N.B. scale on graph of PCB52 and PCB95 is different from the remaining graphs. (a) Results described previously (Antunes Fernandes *et al*., 2010)

Chapter 4

Perinatal exposure to PCB52 and PCB180 increases aromatase activity in rat ovary and adrenal gland and alters gene expression in rat brain at adult age

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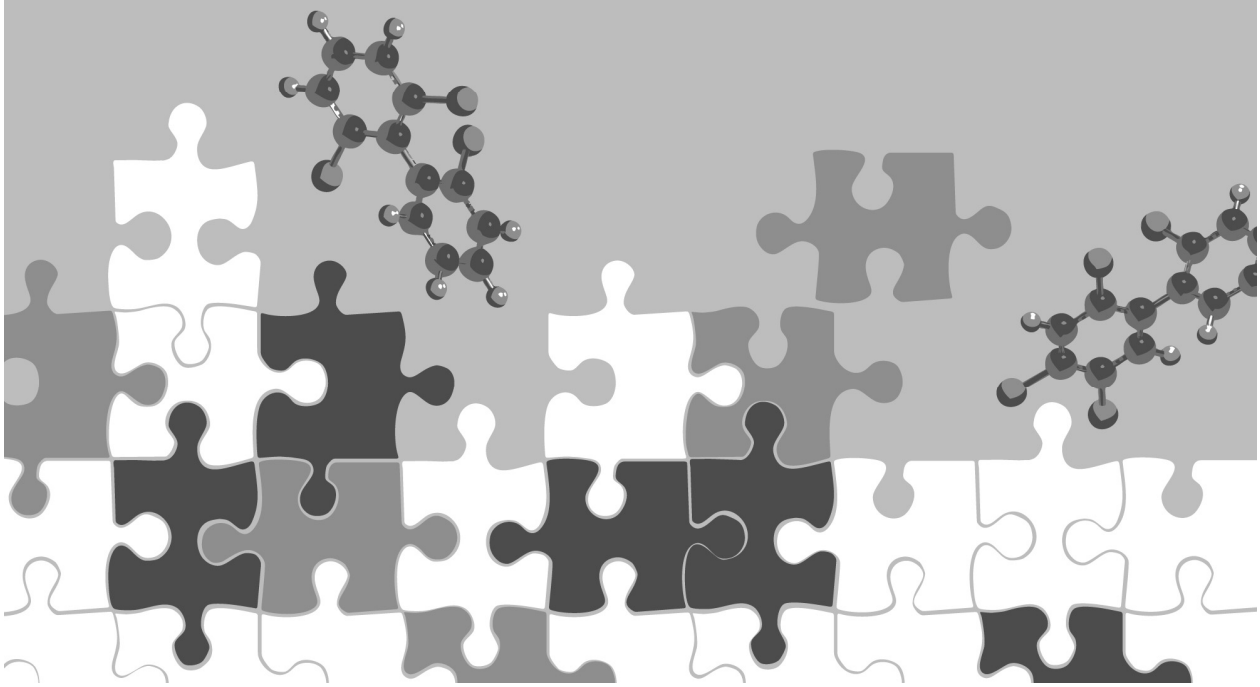

Abstract

The brain, adrenals and gonads constitute an important axis in maintaining sex steroid homeostasis and regulating steroidogenesis during perinatal development and adult life. Non-dioxin-like polychlorinated biphenyls (NDL-PCBs) are ubiquitous environmental pollutants having an endocrine disruptive potential. However, information on the effect of perinatal exposure to single congeners on steroidogenic enzymes (e.g. aromatase) at various life stages is lacking.

Therefore, two perinatal toxicity studies with Sprague-Dawley rats were conducted with the highly-purified NDL-PCB52 and NDL-PCB180. The cumulative doses were 0-3000 mg/kg bw for PCB52 and 0-1000 mg/kg bw for PCB180. At PND 7, 35 and 84, aromatase activity was determined in microsomes of the ovaries, testicals, and adrenals. Perinatal exposure to either PCB180 or PCB52 did not result in an apparent or persistent change in aromatase activity in male rats.. In females on the other hand, perinatal exposure to PCB52 caused a dose-dependent increase in adrenal and ovarian aromatase activity but only at PND84. Gene expression in adult brain of perinatally exposed animals to PCB180 showed that the androgen receptor was down-regulated in females at PND 84. Although the link between NDL-PCB exposure during fetal and neonatal development is difficult to establish, this study suggests that exposure during a critical window of development may cause adverse neuroendocrine effects at adult age.

Introduction

Polychlorinated biphenyls (PCBs) comprise of a group of 209 different congeners, which have been widely used in a number of industrial and commercial applications. Although production and use of PCBs has been prohibited in most countries since the 1980's, these are persistent pollutants that can still be found in the environment and (human) food chain. (European Food Safety Authority (EFSA), 2005). PCBs can be divided into two classes according to their toxicological and chemical properties. Non-*ortho*-substituted congeners are also referred to as dioxin-like PCBs due to their shared toxicological profile with 2,3,7,8-tetrachloordibenzo-p-dioxin (TCDD), which is mediated via the Aryl hydrocarbon receptor (AhR) (Van den Berg *et al.*, 2006, Safe, 1990). *Ortho*-substituted congeners, also referred to as non-dioxin like PCBs (NDL-PCBs), have low affinity for the AhR and produce toxicity via other mechanisms. Environmental and dietary exposure to PCBs occurs to both groups of congeners, However, in spite of the much higher abundancy of NDL-PCBs the information about toxicity or mechanism of action is much more limited than that for DL-PCBs (Breivik *et al.*, 2002).

Several epidemiological studies indicated that background *in utero* and lactational exposure to DL- and NDL-PCBs result in reduced birth weight, less postnatal growth (Rylander *et al.*, 1998), impaired immune development and responses (Weisglas-Kuperus, 1998), and lower thyroid hormone levels (Brouwer *et al.*, 1998, Koopman-Esseboom *et al.*, 1997). Further, levels of PCBs in sera have been related to changes in age of puberty onset (for review see (Schoeters *et al.*, 2008)) and reduced sperm quality (Richthoff *et al.*, 2003). *In vivo* rodent studies with commercial or reconstituted PCB mixtures, composed of DL- and NDL-PCBs, have shown changes in sex-dependent behaviors, such as sweet preference, altered uterine weight (marker for estrogenic activity), reduced testes weight and serum testosterone levels, a sign for antiandrogenic activity (Kaya *et al.*, 2002,Hany *et al.*, 1999). Despite the growing evidence of endocrine toxicity of NDL-PCBs the *vivo* effects appear to be dependent on the timing of exposure and congeners used. So far, there are very few studies focusing on the effects of individual NDL-PCB congeners and their mechanism of action (Schoeters *et al.*, 2008,Dickerson and Gore, 2007). Nevertheless, several *in vitro* studies with single congeners have shown that NDL-PCBs can interact with steroid hormone receptors and these can result in anti-androgenic or weakly estrogenic effects (Portigal *et al.*, 2002,Schrader and Cooke, 2003,Layton *et al.*, 2002). Further, NDL-PCBs have been shown to interact with thyroid hormone metabolism (Kobayashi *et al.*, 2009) and affect the *in vitro* expression of several key enzymes in steroidogenesis, such as aromatase (CYP19) and CYP17 (Xu *et al.*, 2006).

Several experimental studies showed that the placenta is not an efficient barrier to prevent fetal exposure to PCBs (Correia Carreira *et al.*, 2011). Moreover,

due of their lipophilicity and subsequent accumulation in fatty tissues, significant amount of PCBs are transferred to the offspring by breast feeding. Obviously, significant exposure to PCBs occurs during critical developmental life stages, which might increase the risk of hampered development or developing diseases in later life (Colciago *et al.*, 2009). In rodents and humans, the hormonal environment is responsible for brain sex differentiation at early life. In addition, it is also known that steroid hormones and their interactions with steroid receptors continue to modulate brain architecture and function throughout life (Review see (Boon *et al.*, 2010, Hines, 2011)).

Sex steroidogenesis is regulated by the hypothalamic-pituitary-adrenal-gonadal (HPAG) axis at all life stages and strongly coordinates the reproduction process in adult life. An important step in steroidogenesis is the conversion of androstenedione and testosterone into estrone or estradiol by aromatase. Aromatase is present in the gonads, but also in. adrenals, placenta, mammary gland and the brain (Simpson, 2002). However, in regulating steroidogenesis, not only the gonads but also the adrenal gland plays a pivotal role. During fetal development, the adrenals and placenta, or the so-called feto-placental unit, constitute a complete estrogen-synthesizing system. This enzyme system plays a key role in the maintenance of pregnancy, fetal maturation and development and, at least in some species, the initiation of parturition (Ishimoto and Jaffe, 2010).

Various *in vivo* studies have previously been performed to determine endocrine effects of NDL-PCBs, mainly using commercial or reconstituted mixtures. However, these mixtures often contained (low levels) of potent dioxin-like PCBs, which makes it difficult to discern effects of single NDL-PCBs on endocrine or toxicological endpoints, such as steroidogenic enzyme activity and sex steroid receptor expression. In our present study, rats were exposed during fetal development and neonate life to highly purified PCB52 or PCB180, two of the most abundant NDL-PCBs in human food. Aromatase activity in the adrenals and gonads were determined at three different life stages PND7 (neonatal), PND35 (after weaning), and PND84 (adulthood). In addition, effects of perinatal PCB exposure on gene expression of relevant steroid receptors and aromatase were determined in adult rat brain.

Materials and methods

Chemicals

NDL-PCB52 (2,2',5,5'-tetrachlorobiphenyl) and NDL-PCB180 (2,2',3,4,4',5,5'-heptachlorobiphenyl) were purchased from CHIRON (Trondheim, Norway). Possible impurities, e.g., polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/Fs) and DL-PCBs, were removed by M. Stenberg and Dr. P. Andersson

(Institute of Environmental Chemistry, Umeå University, Umeå, Sweden) by applying the PCBs dissolved in n-hexane on an active carbon column and collecting them after elution in n-hexane, as earlier described by Danielsson *et al* (2008).

Animals and treatment

Male and female Sprague-Dawley rats (Harlan Scandinavian) were housed in standard stainless steel cages with wire-mesh cover, in a room with a 12h light/12h dark cycle and controlled temperature (21±2 ^{o}C). Aspen chips (FinnTapvei, Kaavi, Finland) were used as bedding and nesting material. Pelleted laboratory animal feed (R36, Lactamin, Sweden) and water were available *ad libitum*.

Adult females (12-15weeks old) were mated with males in the same age range. The day after the mating, when sperm was found in vaginal smears, was considered day 0 of gestation (GD0). Pregnant rats were orally exposed to four equal doses of vehicle or PCB180 dissolved in corn oil, to a total dose of 0, 0.3 and 1 g/kg bw. Pregnant and lactating dams were exposed to ten equal doses of vehicle or PCB52 dissolved in corn oil to a total dose of 0, 1 and 3 g/kg bw. The scheme of dosing and sacrifice of the animals are shown in figure 1.

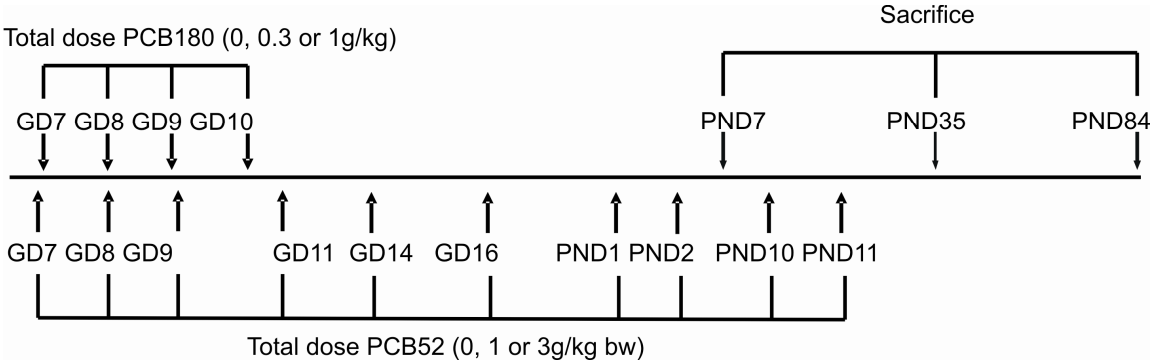

Figure 1. Overview of animal exposure to PCB52 and PCB180, and the sacrifice days.

Animals were sacrificed at postnatal day (PND) 7, 35 or 84, either by decapitation at PND7 or by CO_2 asphyxiation and exsanguination on later time points. Adrenal glands, ovaries, testis and brain (cerebrum and cerebellum) were removed, snap frozen in liquid nitrogen and stored at -80^{o}C prior to use. All experiments were approved by the animal experiment committee of Finland.

Preparation of tissues

The ovaries, testis and adrenal glands were used to isolate microsomal fractions and used for further analyzes. Frozen tissues were weighed and

homogenized in an Potter-Elvehjem device with 10 volumes of TRIS-HCl buffer (TRIS-HCl 50mM; 1.15%KCl), at a pH of 7.4 and centrifuged at 10000 rpm during 25min at 4°C. Supernatant was removed and centrifuged again at 30000 rpm during 1h15min at 4°C. Supernatant (cytosol) was decanted and the pellet (microsomes) was resuspended in a sucrose solution (0.25M). Protein content was determined according to Lowry *et al* (1951).

RNA isolation

RNA was isolated from brain tissue (cerebrum and cerebellum) using RNA InstaPure according to supplier's instructions (RNA InstaPure™ Eurogentec, Liège, Belgium). RNA purity and concentration were determined spectophotometrically at wavelengths of 230, 260 and 280nm. Purified RNA was kept at -80°C prior to use.

Aromatase activity

The catalytic activity of aromatase in microsomes was determined by the tritiated water release method, as previously described (Lephart and Simpson 1991). Briefly, microsomes (5–10 mg protein/mL) were incubated with 357nM of [1β-^{3}H] androstenedione (PerkinElmer Life Sciences Inc.) dissolved in HEPES/$MgCl_2$ buffer (50mM/5mM, pH 7.8) during 1h at 37°C and 5% CO_2. The next steps were conducted as described before (Sanderson 2001, Letcher, 1999). Hydroxyandrostenedione (4-HA, 0.25mM) was used as a positive control for aromatase catalytic inhibition (Heneweer *et al.*, 2005). Corrections were made for background radioactivity and dilution factor. Of each pooled sample three measurements were performed; one for background and two separate measurements for aromatase activity. Aromatase activity of each sample was determined as average of the two measurements subtracted by the background.

Only in ovaries and testis of animals sacrificed at PND84, aromatase activity was determined in individual samples. Due to small sample sizes, the aromatase activity in adrenals and gonadal tissues at PND 7 and PND 35 could only be determined using pooled samples. In this case individual tissues from 4 to 8 animals were pooled. From each pooled sample two measurements of aromatase activity were performed and one microsomal aliquot was used for background correction. As a result no statistical analyses could be performed on the pooled samples.

Determination of gene expression by real time quantitative polymerase chain reaction (RT-qPCR)

RNA was diluted to a concentration of 66.7 µl/ml with RNAse free water. Reverse transcription was performed on 1µg of RNA, in a final volume of 20 µl, with

iScript cDNA Synthesis Kit, according to the manufacturer's instructions (BioRad, Veenendaal, The Netherlands). Per sample, a PCR mix was made containing 12.5 µl of iQ SYBR Green Supermix, 0.4 µM of each primer (forward and reverse) and 0.5 µL RNAse free water. For the RT-qPCR 15 µl of this mixture was was added to 5 µl of cDNA of each sample. Table 1 gives an overview of the oligo sequence of the genes studied in the present study. β-Actin was used as the house hold gene.

Table1. Sequence of the primers used for the RT-PCR experiments

Gene name	Oligo sequence	
	Forward primer	Reverse primer
CYP19	GCCTGTGGAGAACGGTCCGC	ACCACGTCCACGTAGCCCGA
ERα	AAGCTGGCCTGACTCTGCAG	GCAGGTCATAGAGAGGCACGA
ERβ	CTCTGTGTGAAGGCCATGAT	GGAGATACCACTCTTCGCAATC
AR	CACCATGCAACTTCTTCAGCA	CGAATTGCCCCCTAGGTAACT
β-Actin	AGCGTGGCTACAGCTTCACC	AAGTCTAGGGCAACATAGCACAGC

Statistical analyses

Statistical significant differences among groups in which samples were not pooled, including gene expression data were determined by student t-test. Differences were considered statistically significant if $P<0.05$.

Results

Effect of PCB52 on adrenal aromatase activity.

Basal aromatase activity in male adrenal microsomes was low from PND7 to adulthood. Activity was comparable at PND7 and PND84, 0.12 and 0.07 pmol/h/mg protein, respectively. Although low in male pups, aromatase showed the highest activity (0.42 pmol/h/mg protein) on PND35. At this age, male pups exposed to either 1000 or 3000 mg/kg bw of PCB52 showed a dose-dependent decrease of aromatase activity to 0.07 and 0.02 pmol/h/mg protein, respectively (Figure 1A). In the male pups dosed with 1000 and 3000 mg/kg bw there was an apparent decrease in aromatase activity with the increasing age of the pups (Figure 1A).

In female pups, basal aromatase activity in adrenal microsomes increased with the age of the female pups, from 0.02 pmol/h/mg protein at PND7 to 0.39 pmol/h/mg protein at PND84. Further, at PND84, female offspring exposed to 1000 and 3000 mg/kg bw showed a dose-dependent increase in aromatase activity to 4.00

and 7.11 pmol/h/mg protein, respectively. At PND35, aromatase activity in adrenals was approximately the same in female offspring that had been perinatally exposed to 0 and 1000 mg/kg bw, 0.07 and 0.03 pmol/h/mg protein respectively. Further, at PND35 in the female offspring exposed to 3000 mg/kg bw the aromatase activity could not be detected at PND35 (Figure 1B).

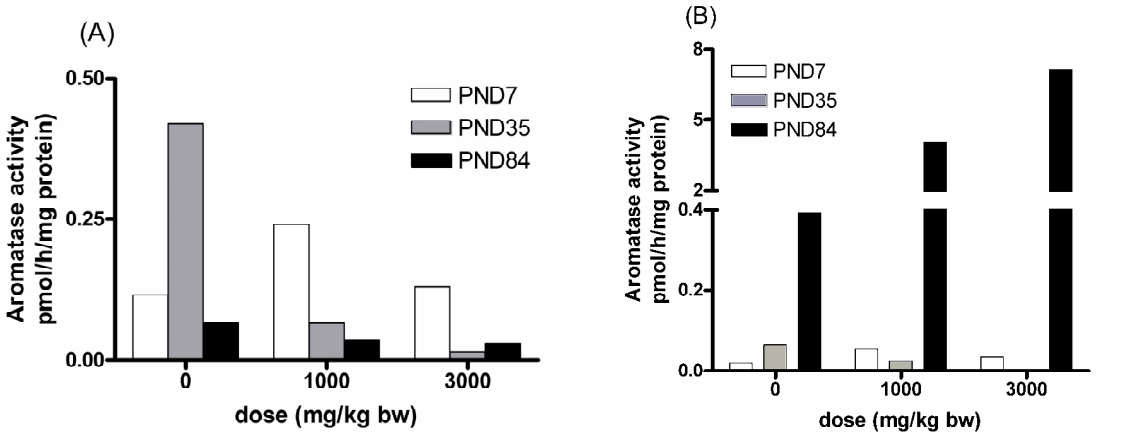

Figure 1. Aromatase activity in adrenal microsomes isolated from male (A) and female (B) pups. Dams were dosed with 0, 1000 and 3000 mg/kg bw of PCB52 and pups were sacrificed at PND7, PND35 and PND84. Data represents pooled tissues per group, consisted of four to seven animals.

Effect of PCB180 on adrenal aromatase activity.

Exposure to PCB180, at different doses, did not induce any apparent changes in aromatase activity in female adrenals, at any of the PND's tested. Although aromatase activity in male adrenal microsomes showed to be decreased with age, this was only observed in animals exposed to 1000 mg/kg bw. This age-dependent decrease in aromatase activity, was not observed in female adrenal microsomes (cf. Figure 2A and 2B). At PND7, aromatase activity in male adrenal microsomes of control animals could not be detected. However, at PND35 and PND84 aromatase activity was 0.05 and 0.04 pmol/h/mg protein, respectively. At PND7, animals perinatally exposed to 300 and 1000 mg/kg bw of PCB180 showed a slight dose-dependent increase in aromatase activity (to 0.11 and 0.17 pmol/h/mg protein, respectively). However, at PND35 and PND84 this apparent dose-dependent effect of PCB180 in male adrenal aromatase activity was no longer observed.

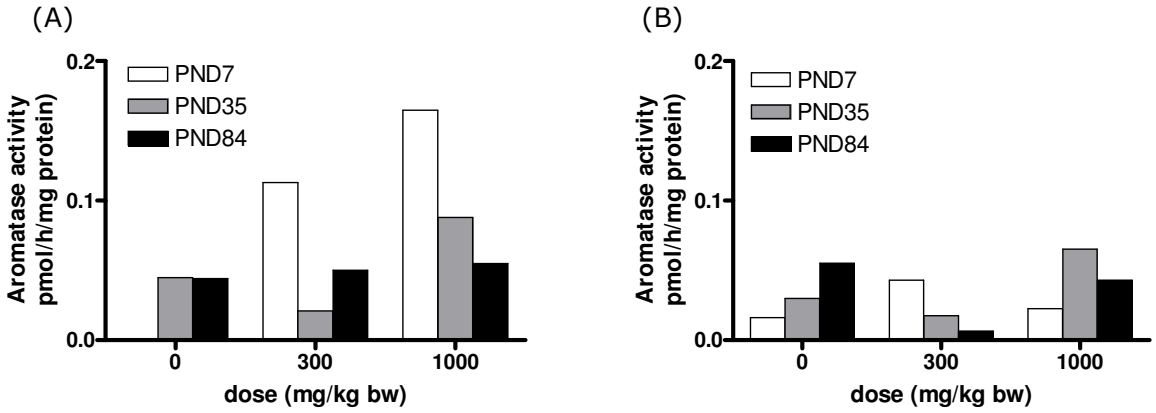

Figure 2. Aromatase activity in adrenal microsomes isolated from male (A) and female (B) pups. Dams were dosed with 0, 300 and 1000 mg/kg bw of PCB180 and pups were sacrificed at PND7, 35 and 84. Data represents pooled tissues per group, consisted of four to seven animals.

Effect of PCB52 and PCB180 on ovarian aromatase activity.

At PND7, exposure to PCB52 showed no apparent effect on aromatase activity in ovarian microsomes. At PND35 animals exposed to 0 and 1000 mg/kg bw of PCB52 showed similar aromatase activity as the control animals, 0.82 and 0.73 pmol/h/mg protein, repectively. However at the higher dose, 3000 mg/kg bw, aromatase activity was decreased to 0.06 pmol/h/mg protein. At PND84, exposure to PCB52 caused a dose-dependent increase in aromatase activity, from a basal level of 0.48 pmol/h/mg protein to 2.20 pmol/h/mg protein (1000 mg/kg bw) and 3.97 pmol/h/mg protein (3000 mg/kg bw). Especially in the highest dose group, an age-related increase of aromatase activity could be observed.

Aromatase activity in ovarian microsomes isolated from animals exposed to PCB180 was affected differently than observed for PCB52. At PND7 basal aromatase activity was low (0.02 pmol/h/mg protein) and it was induced in females exposed to 300 and 1000 mg/kg bw to 0.13 and 0.31 pmol/h/mg protein, respectively. When compared to PND7, ovaries of females sacrificed at PND35 and PND84 showed a significant increase in basal aromatase activity (3.39 and 6.85 pmol/h/mg protein respectively). At PND35 and PND84 induction of aromatase activity was the strongest at 300 mg/kg bw. At a higher dose, aromatase activity was reduced to values comparable control activity.

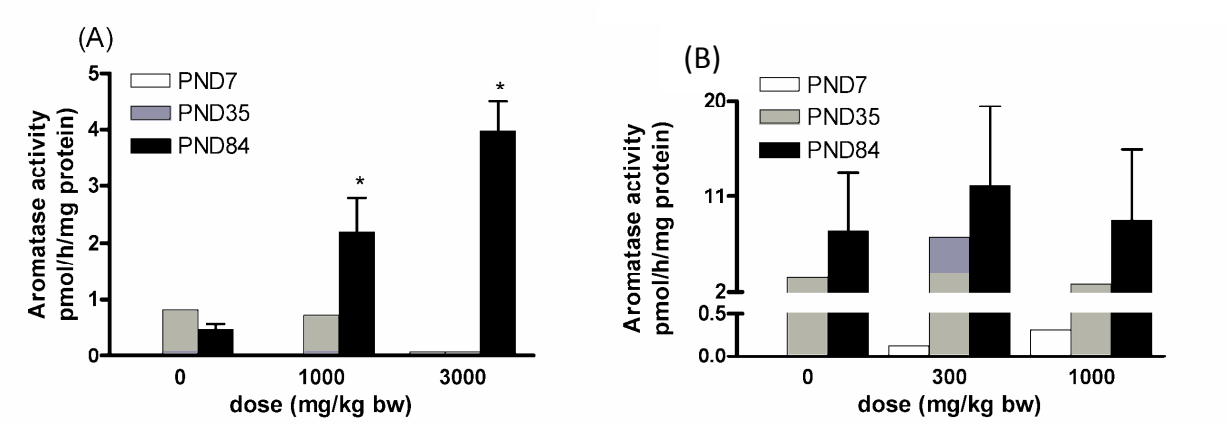

Figure 3. Effect of PCB52 and PCB180 on aromatase activity in rat ovaries. Dams were dosed with 0, 1000 and 3000 mg/kg bw of PCB52 (A) or with 0, 300 and 1000 mg/kg bw of PCB180 (B). Animals were sacrificed at PND7, PND35 and PND84. Data represents pooled tissues per group, except tissues isolated from animals at PND84, consisted of four to seven animals. (*p<0.05 *vs* control animals)

Effect of PCB52 and PCB180 on aromatase activity in testis microsomes.

At PND7 aromatase activity in testicular microsomes was not measurable in animals exposed to PCB52. At PND35, low aromatase activity (0.02 pmol/h/mg protein) could only be measured in animals exposed to 1000 mg/kg bw. At an older age (PND84) aromatase activity increased to 0.14 pmol/h/mg protein, however this activity was apparently not affected by exposure to different doses of PCB52.

Aromatase activity in microsomes isolated from testes was very low, independent of the age of the pups and exposure dose of PCB180. Further, at PND7 aromatase activity could not be detected in microsomes isolated from testicular microsomes, independent of the dose of exposure. At PND35 aromatase activity of control animals was low (0.03 pmol/h/mg protein) and further decreased with increasing dose of PCB180. However, there was a slight age-dependent increase in aromatase. Still low (0.05 pmol/h/mg protein), at PND84, aromatase activity was measurable. Exposure to different doses of PCB180 did not have any apparent effect on this activity.

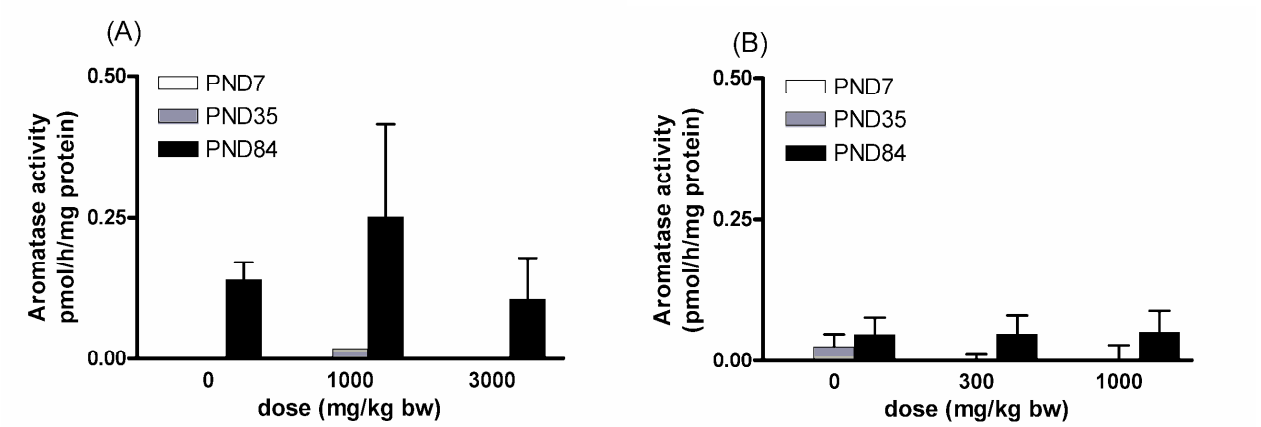

Figure 4. Effect of PCB52 and PCB180 on aromatase activity in rat testis. Dams were dosed with 0, 1000 and 3000 mg/kg bw of PCB52 (A) or with 0, 300 and 1000 mg/kg bw of PCB180 (B). Animals were sacrificed at PND7, PND35 and PND84. Data represents pooled tissues per group, except tissues isolated from animals at PND84, consisted of four to seven animals.

Effect of exposure to PCB52 and PCB180 on gene expression in rat brain

Expression of brain CYP19 (aromatase) was slightly, yet not significantly, up-regulated in both male and female rats exposed to 3000 mg/kg bw of PCB52 and in male rats exposed to 1000 mg/kg bw of PCB180. Expression of the estrogen receptor alpha (ERα) and ER beta (ERβ), was not altered by perinatal exposure to either PCBs. Expression of androgen receptor was significantly (*p<0.05) decreased in females exposed to 1000 mg/kg bw, but not in males.

Table 2. Gene expression of aromatase (CYP19), estrogen receptor alpha (ERα) and beta (ERβ), and androgen receptor (AR) in rat brain (cerebrum and cerebellum). Dams were dosed with 3000 mg/kg bw of PCB52 or with 1000 mg/kg bw of PCB180. Pups were sacrificed at PND84. Data represents average per group, consisted of four to seven animals. Results are represented as ratio of gene expression of exposed animals in comparison to gene expression of control animals (± SD). (*p<0.05 *vs* control animals)

	mg/kg	CYP19	Erα	ERβ	AR
PCB52 ♂	3000	2.5 (± 3.1)	0.9 (± 0.2)	1.1 (± 0.2)	0.9 (± 0.3)
PCB52 ♀	3000	2.5 (± 1.7)	0.8 (± 0.3)	0.8 (± 0.3)	1.0 (± 0.1)
PCB180 ♂	1000	1.6 (± 0.5)	1.3 (± 0.5)	0.9 (± 0.1)	1.3 (± 0.1)
PCB180 ♀	1000	1.0 (± 0.6)	1.2 (± 0.2)	1.2 (± 0.2)	*0,61 (± 0.1)

Discussion

Although several studies suggest the potentially endocrine toxic effects of NDL-PCBs, the variations in study design, endpoints and purity of congeners make

interpretation and comparison of these studies difficult (Kobayashi *et al.*, 2009,Khan *et al.*, 2002,Jansen *et al.*, 1993). In our study, we investigated the effects of perinatal exposure to two NDL-PCBs, which are from a quantitative point of view among the most common PCBs in food-chain. The brain, adrenals and gonads constitute an important axis in maintaining sex steroid homeostasis and regulating steroidogenisis during fetal and neonatal development, but also later in adult life. In order to assess potential changes in this axis due to exposure to NDL-PCBs during fetal and neonatal development or adulthood, we studied the activity of a key enzyme in steroidogenesis, aromatase, at these different life stages of the rat. *In vivo* studies have shown that exposure to mixtures DL- and NDL-PCBs of can regulate its aromatase activity and expression (Hany *et al.*, 1999,Colciago *et al.*, 2009). However, insight on the effects of exposure to single NDL-congeners id still lacking. Further, gene expression of steroid hormone receptors (ERα, ERβ and AR) and aromatase in the adult brain after perinatal exposure was measured.

In the human fetal adrenal gland, sex steroid hormone synthesis is favored while in the adult adrenal, production of glucocorticoids and aldosterone becomes more important. In this study, an age-dependent increase in adrenal aromatase activity was observed in control rat females. In males this effect was less pronounced and aromatase activity even appeared to decrease with age. This gender difference does not appear to exist in postnatal adrenal androgen secretion in humans, as DHEA and androstenedione secretion sharply decrease in the first week of life of both males and females (Ben-David *et al.*, 2007). Although in general male androgen plasma levels increase as of day 21 and these are higher in newborn males than females, due to increased testicular androgen production. This is in agreement with the age-dependent increase in testicular and ovarian aromatase activity we observed in our study.

Perinatal exposure to PCB180 did not result in a significant change in aromatase activity in the steroidogenic axis of male and female rats. This also appears to be the case for perinatal exposure to PCB52 in males. In males, a moderate dose-dependent decrease in adrenal aromatase activity was observed by PCB52 only at PND35, but no effect was seen on testicular aromatase activity. In females on the other hand, perinatal exposure to PCB52 caused a dose-dependent increase in adrenal aromatase activity in adult female rats (at PND84), but not at younger ages. In addition, a dose-dependent increase in ovarian aromatase activity by PCB52 was observed at PND84. This suggests that exposure to PCB52 during fetal development and neonatal life might affect aromatase activity in female steroidogenic axis in adult life. In this respect it should also be noticed that females might be more vulnerable for disruption of the steroidogenic system, because their neonatal steroid hormone levels are lower than that of males (Bakker and Brock, 2010). In addition*, in vivo* experiments of male and female adult rats exposed to brominated flame retardant (BFRs). show that females have a lower metabolism. This

results in a higher systemic exposure of the parent compound compared to male tissues, which may explain the sex-different toxic effects. (van der Ven *et al.*, 2009, Canton *et al.*, 2008). Estrogen production in ovaries is among others responsible for the up-regulation of other receptors involved in the ovulation. Interaction between xenobiotics and aromatase may have significant implications with various important physiological or endocrine processes. Firstly, any interference with estrogen production could interact with the natural ovulation process, thereby directly influencing the reproduction process (Clarkson and Herbison, 2009). In addition, cumulative life-time exposure to high levels of estrogens is associated with increased breast cancer risk. Some epidemiological studies have associated PCB52 levels in breast fat with breast cancer and the results from our study could provide a biological plausibility for the observation (Lucena *et al.*, 2001). However, it must be noted that the dose levels of the PCBs used in our study were very high and do actually reflect background human intake levels. Generally, the majority of human exposure occurs through food intake and the daily intake of NDL-PCBs is estimated to be around 10-45 ng/kg bw per day. Therefore, the practical meaning of our results on aromatase interaction by these PCBs might be questionable.

It has frequently been suggested that exposure to different classes of xenobiotics, such as PCBs, BFRs or synthetic steroids, during fetal and neonatal development can result in persistent physiological changes leading to adverse effects in adulthood (Colciago *et al.*, 2009, van der Ven *et al.*, 2009, Canton *et al.*, 2008, Tegethoff *et al.*, 2009). Changes in the steroidogenic axis might result in altered circulating hormone levels, but also can affect gene expression of e.g. steroidogenic enzymes or steroid hormone receptors. In recent years, attention has focused on aromatase in the male hypothalamus, at young age, due to its importance in brain masculinization. The role of aromatase in female brain is not yet fully understood (Bakker and Brock, 2010, Wu *et al.*, 2009, Gagnidze and Pfaff, 2009). Further, aromatase is not only expressed in hypothalamus but also in other brain areas, which are important for different functions, such as motor activity or leaning and memory. At later life stages, the function of aromatase in the brain seems to adopt to a more neuroprotective effect. The results of our study suggest an increase in aromatase expression in adult cerebrum and cerebellum as a result of perinatal PCB exposure, but from a statistical point of view these were no unequivocal. The importance of aromatase in brain tissue is also indicated from the fact that estrogens synthesize in the hippocampus are involved in the regulation of synaptic plasticity and neurogenesis (Kretz *et al.*, 2004, Tanapat *et al.*, 2005). Interestingly, female rats from our study that were exposed to PCB180 showed a decreased gene expression of the AR in the brain. In spite of large molecular size, it has been shown that PCB180 can indeed cross the blood-brain barrier (Kodavanti *et al.*, 1998). In addition, PCB180 has a half-life of 81 days (Oberg *et al.*, 2002) and many of its metabolites are detectable in human samples and even appear to be more active than the parent compound

when binding to the thyroid hormone receptor (Kitamura *et al.*, 2005). Our observations are in line with several other studies, which have also shown a decrease in brain AR expression combined with a masculinization/defeminization of sexual differentiation of the female neuroendocrine systems upon perinatal exposure to NDL-PCBs (Colciago *et al.*, 2009,Dickerson *et al.*, 2011).

In summary our experiments with two environmental relevant NDL-PCBs indicate that these can modulate the aromatase activity in different tissues of the rat. Results were tissue, sex, age as well as congener specific. The latter observations are not surprising, as expression of aromatase is highly sex and age dependent. The results of our study indicate that prenatal exposure to some NDL-PCBs can change the expression of this key steroidogenic enzyme and may persist in adulthood. However, it must be noted that the levels at which modulation of aromatase was seen were several orders of magnitude above that of the general human background exposure. In view of the differences observed between PCB 52 and PCB180, further studies must shown what type of structural requirements are essential for these aromatase interactions, besides elucidating the possible role of metabolites.

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Chapter 5

OH-PCBs are more potent aromatase activity inhibitors and (anti-) glucocorticoids than PCBs and $MeSO_2$-PCBs

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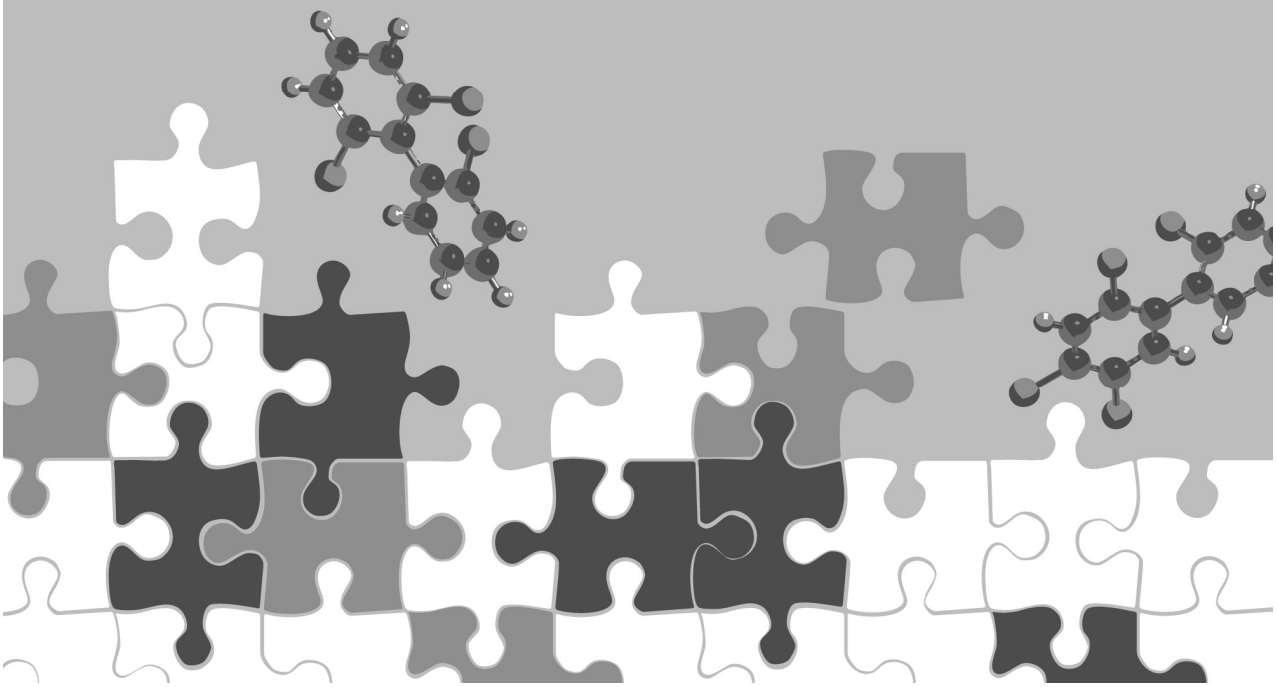

Abstract

Traditional risk assessment of potential endocrine-disruptive pollutants, including PCBs, focus mainly on the effects of parent compounds. Still, biotransformation results in systemic exposure to PCBs and their bioactive metabolites. In the present paper, the effects of twenty non-dioxin-like (NDL) PCBs and their environmentally relevant hydroxy- (OH-) and methylsulfonyl- ($MeSO_2$-) metabolites on aromatase activity in human placental microsomes and their glucocorticoid properties were investigated.

Although most NDL-PCBs were inactive, PCB28 inhibited aromatase activity with an IC_{50} of 2.2 μM. Most of these NDL-PCBs were weak (ant-)agonist of the glucocorticoid receptor (GR). Interestingly, four OH-metabolites of the commonly found NDL-PCB180 were able to inhibit aromatase activity (LOECs in the low μM range) and showed anti-glucocorticoid properties (LOECs in the low nM range), in a concentration dependent manner. Further, four $MeSO_2$-PCBs slightly inhibited aromatase activity and showed anti-glucocorticoid properties. Although, these effects were also associated with cytotoxicity, they were dependent on the position of the $MeSO_2$-group on the biphenyl ring.

Our results are the first showing that OH-PCBs are both anti-glucocorticoids and aromatase inhibitors. Taken together, these results for PCBs again support the common idea that risk assessment of the endocrine disruptive potential of PCBs should also include their metabolites.

Introduction

Production and commercialization of polychlorinated biphenyls (PCBs) has been largely prohibited since the 1970's. Still PCBs are ubiquitous pollutants as their chemical stability, persistency and lipophilicity result in their tendency to bioacumulate in food chain, wild life, environment and humans.

In animals, biotransformation of PCBs into hydroxy-PCBs (OH-PCBs) involves oxidation by cytochrome P-450 (CYP-450) enzymes, resulting in more water soluble congeners. However, not all the OH-PCBs are excreted but further metabolized into methylsulfonyl-PCBs ($MeSO_2$-PCBs) involving additional CYP-450 monooxygenation and phase II conjugation (for review see Letcher *et al.*2000). The hydroxy or methylsulfonyl group attached to the biphenyl backbone circumstantiate different toxicological characteristics. In fact, the difference in lipophilicity of OH- and $MeSO_2$-PCBs results in these two classes of metabolites accumulating and exerting their effects in different target tissues. OH-PCBs can be found in human and animals at concentrations as high as 10%-40% of the total PCB concentration in human serum, which can be as high as 268 ng/g lipid (Bergman *et al.* 1994; Sandanger *et al.* 2004; Sandau *et al.* 2000; Sjodin *et al.* 2000). These metabolites are hardly retained in the lipid fraction of tissues, but bind to blood proteins such as transthyretin, which facilitates OH-PCBs passing to the blood cord (Morse *et al.* 1995). Further, several studies have reported that OH-PCBs are present in human cord blood in similar concentrations as their parent compounds (Park *et al.* 2008), whereas $MeSO_2$-PCBs are only found in much lower amounts in human blood (Linderholm *et al.* 2007). In contrast, $MeSO_2$-PCBs tend to accumulate in human liver, lung, adipose tissue and breast milk, and concentrations can be as high as 358 ng/g lipid, depending on the tissue (Chu *et al.* 2003; Weistrand and Noren 1997).

Today, the existence and occurrence of OH-PCBs and $MeSO_2$-PCBs in biota has been widely described, but their toxicological characteristics are still largely unknown.

PCBs are a class of pollutants composed of 209 congeners, which according to their chemical and toxicological properties can be divided into dioxin-like PCBs (NDL-PCBs) and non-dioxin-like PCBs (NDL-PCBs) (Van den Berg *et al. 2005)*. The main route of human exposure to NDL-PCBs is through food, although recent studies also direct toward house dust (Abb *et al. 2*010; Harrad *et al.* 2010). In 2005 the European Food Safety Authority (EFSA) has identified six common NDL-PCBs (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180) which together account for more than 50% of the total NDL-PCBs found in food and feed (EFSA 2005).

In utero exposure to NDL-PCBs has been related to retarded postnatal growth, and neurodevelopment deficits in humans. However, it is not clear how much of these effects are caused by the parent compounds or their metabolites (Patandin *et al.* 1998; Stewart *et al.* 2003). Some NDL-PCBs have been shown to

interact with endocrine pathways, for example by binding and/or activating steroid hormone receptors or interfering with key enzymes in sex steroidogenesis (Kraugerud *et al. 2010*). In sex steroidogenesis, aromatase plays a key role, being responsible for the aromatization of androgens into estrogens. This enzyme is expressed in various tissues, such as the human placenta. In rodents a disruption of aromatase function during the fetal masculinization period has been shown to alter the brain function later in life (Hany *et al.* 1999). Various mechanisms are involved in the regulation of aromatase expression and activity and due to alternative expression of different promoters, tissue-specific aromatase expression is achieved. In adipose tissues, skin and fetal liver, aromatase expression is mainly promoter I.4, I.3 and II-driven, of which PI.4 is regulated by glucocorticoids (for review see (Kamat *et al.* 2002)). In addition, there are a few studies suggesting that both PCBs and $MeSO_2$-PCBs can interact directly with the glucocorticoid pathway through the glucocorticoid receptor (GR), but little is known about the affinity of OH-PCBs with this receptor (Johansson *et al.* 1998a; Johansson *et al.* 1998b).

In this study, the effects on aromatase activity and GR activation of twenty selected NDL-PCBs and their most relevant OH- and $MeSO_2$-metabolites have been studied in human placental microsomes, human adrenocortical carcinoma cells (H295R) and a recently developed yeast glucocorticoid bioassay, respectively.

Selection of NDL-PCBs was based on their chemical and physical properties, environmental and biological abundance and toxicological properties (Stenberg and Andersson 2008). The choice of the OH- and $MeSO_2$- metabolites was based on their environmental abundance. Further, the tested OH-PCBs represent four possible metabolites of the commonly found NDL-PCB180.

Material and methods

Chemicals

The twenty tested PCBs (Table 1), were purchased from Neosync Inc., USA. Possible impurities, e.g., polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/Fs) and DL-PCBs, were removed by applying the PCBs dissolved in *n*-hexane on an active carbon column and collecting them after elution with *n*-hexane as described by Danielsson *et al* (2008). The four OH-PCBs (Table 1) were custom synthesized by AccuStandard Inc., USA. The four $MeSO_2$-PCBs (Table 1) were synthesized in the Department of Environmental Chemistry, Wallenberg Laboratory, University of Stockholm. Testosterone, Dexamethasone and Budesonide were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). PCBs, PCB metabolites, testosterone, dexamethasone and budesonide were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of stock solutions of PCB (and metabolites) were 10 mM.

Table 1 – Overview of the congeners used in the present study. Inclusion criteria of the NDL-PCBs are described in (Stenberg and Andersson 2008).

Congener	Full name
PCB19	2,2’,6-Trichlorobiphenyl
PCB28	2,4,4’-Trichlorobiphenyl
PCB47	2,2’,4,4’-Tetrachlorobiphenyl
PCB51	2,2’,4,6’-Tetrachlorobiphenyl
PCB52	2,2’,5,5’-Tetrachlorobiphenyl
PCB53	2,2’,5,6’-Tetrachlorobiphenyl
PCB74	2,4,4’,5-Tetrachlorobiphenyl
PCB95	2,2’,3,5’,6-Pentachlorobiphenyl
PCB100	2,2’,4,4’,6-Pentachlorobiphenyl
PCB101	2,2’,4,5,5’-Pentachlorobiphenyl
PCB104	2,2’,4,6,6’-Pentachlorobiphenyl
PCB118	2,3’,4,4’,5-Pentachlorobiphenyl
PCB122	2’,3,3’,4,5,-Pentachlorobiphenyl
PCB128	2,2’,3,3’,4,4’-Hexachlorobiphenyl
PCB136	2,2’,3,3’,6,6’-Hexachlorobiphenyl
PCB138	2,2’,3,4,4’,5’-Hexachlorobiphenyl
PCB153	2,2’,4,4’,5,5’-Hexachlorobiphenyl
PCB170	2,2’,3,3’,4,4’,5-Heptachlorobiphenyl
PCB180	2,2’,3,4,4’,5,5’-Heptachlorobiphenyl
PCB190	2,3,3’,4,4’,5,6,-Heptachlorobiphenyl
4'OH-PCB172	4'OH-2,2',3,3',4,5,5'-Heptachlorobiphenyl
3'OH-PCB180	3'OH-2,2',3,4,4',5,5'-Heptachlorobiphenyl
3'OH-PCB182	3'OH-2,2',3,4,4',5,6'-Heptachlorobiphenyl
5OH-PCB183	5OH-2,2',3,4,4',5',6-Heptachlorobiphenyl
3MeSO$_2$-PCB101	3MeSO$_2$-2,2’,4,5,5’-Pentachlorobiphenyl
4MeSO$_2$-PCB101	4MeSO$_2$-2,2’,4,5,5’-Pentachlorobiphenyl
3MeSO$_2$-PCB149	3MeSO$_2$-2,2’,4’,5,5’,6-Hexachlorobiphenyl
4MeSO$_2$-PCB149	4MeSO$_2$-2,2’,4’,5,5’,6-Hexachlorobiphenyl

Human placenta microsome fraction

Human placenta was obtained from the hospital (St. Antonius Hospital, Nieuwegein, The Netherlands) with informed consent of the patient (TME/Z-02-09, Medical Ethical Committee, St. Antonius Hospital, Nieuwegein, The Netherlands) and stored at -80˚C until analysis. Microsomal fraction was isolated from human tissue by homogenization of samples in 10 volumes of TRIS-HCl buffer (TRIS-HCL 50mM, 1.15% KCl) using a Potter-Elvehjem glass homogenizer. After centrifugation for 25 minutes at 15,000 rpm at 4˚C, the supernatant was centrifuged again for 1.15 hr at 47,000 rpm at 4˚C. Then, the supernatant was decanted and the pellet was resuspended in sucrose solution (0.25 M). The microsome suspension was frozen in aliquots and stored at -80˚C until use. Protein content was measured according to the method of Lowry using bovine serum albumine as protein standard (Lowry *et al.*1951).

H295R cell culture and exposure

Human adrenal carcinoma cell line H295R was obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 1:1 Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12, GibcoBRL 31300-038). Cell culture medium was supplemented with 1% ITS-G (GibcoBRL 41400-045), containing 6.7 µg/l sodium selenite, 10 mg/ml insulin and 5.5 ml/l transferring; 100U/ml penicillin/streptomycin (GibcoBRL 15140-114) and 2% steroid-free replacement serum Ultroser SF (Sopachem, France).

For measuring aromatase activity, $2x10^5$ cells were plated in a 24-wells plate and allow to attach for 24 hours. Then, the culture medium was changed and cells were exposed to PCBs or OH-PCBs (maximum solvent concentration was 0.1% v/v) for 24 hours.

As a positive control for aromatase induction, cells were exposed to 100 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, The Netherlands). 10 µM of 4-Hydroxyandrostenedione (4-HA) was used as a positive control for aromatase catalytic inhibition (Heneweer *et al.* 2004). Cell culture and exposure were performed at 37°C in a humidified atmosphere (95%) at 5% CO_2.

Aromatase assay

The catalytic activity of aromatase was determined in human placental microsomes and H295R cells based on the tritiated water-release method of (Lephart and Simpson 1991) with modifications as described by (Sanderson *et al.* 2001). The aromatization of the substrate [1β-^{3}H]-androstenedione produces ^3H_2O (tritiated water), which is determined as measure for aromatase activity. The specificity of the aromatase assay was verified using 4-HA. The NDL-PCBs were tested

at a high (10 µM) and low (1 µM) concentration. The PCBs that showed an inhibition of the aromatase activity were further tested at a concentration range of 10 nM to 25 µM. OH-PCBs and $MeSO_2$-PCBs were tested at a concentration range of 6.8 nM to 27.5 µM and 1.2 to 12 µM, respectively.

The yeast glucocorticoid bioassay

The yeast glucocorticoid bioassay is based on the yeast cell previously used to construct the yeast estrogen and androgen bioassays (Bovee *et al.* 2007; Bovee *et al.* 2004). In short, the yeast *Saccharomyces cerevisiae* was transformed with a vector containing hormone responsive elements (HRE) in front of the yeast enhanced green fluorescent protein marker gene (p406-HRE2-CYC1-yEGFP-Ura) (Bovee *et al.* 2007). For the GR-bioassay it was additionally transformed with a vector containing the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter in front of the full length cDNA coding for the human glucocorticoid receptor (p403-GPD-hGRα-His). The GR bioassay was shown to be highly specific for glucocorticoids, such as dexamethason and budesonide, and gave no induction of response upon androgen and estrogen exposure (up to 400 µM of testosterone and androstenedione, and 1000 µM of estradiol. Data not shown) The construction and characteristics of this new yeast glucocorticoid bioassay will be described in more detail elsewhere.

Cultures of this yeast glucocorticoid biosensor were grown overnight at 30°C with vigorous orbital shaking in selective minimal medium containing yeast nitrogen base without amino acids and ammonium sulfate, dextrose, and bacto agar (Becton, Dickinson & Co, Sparks, MD, U.S.A.) and supplemented with L-leucine (MM/L) (Sigma-Aldrich, Steinheim, Germany). At the late log phase, the culture was diluted in MM/L to an optical density (OD at 630 nm) between 0.04 and 0.06. For exposure, aliquots of 200 µl of this diluted yeast culture were pipetted into each well of a 96-well plate and 2 µl of a stock solution in DMSO was added to test the agonistic properties of the compounds. To test for anti-(gluco)corticoid properties, 1 or 2 µl of the stock solutions were co-exposed with either 1 µl of budesonide or dexamethasone stock solutions known to cause a half-maximal effect (EC_{50}). DMSO and controls containing only budesonide or dexamethasone were included in each experiment and each sample concentration was tested in triplicate. Exposure was performed for 24 h at 30°C and orbital shaking (125 rpm). Fluorescence and OD were measured at 0 and 24 h directly in FLUOstar Galaxy (BMG Labtechnologies) using excitation at 485 nm and emission at 530 nm for the fluorescent measurement. The fluorescent signal was corrected with the signals obtained with the supplemented MM/L containing DMSO solvent only. In order to check if a substance was toxic to the cells, the density of the yeast culture was determined by measuring the OD at 630nm (Bovee at al 2004).

Data analysis

Experiments were performed in duplicate, and within each individual experiment each concentration was tested in triplicate. All results are presented as mean of all pooled measurements, ± SD (n=6, unless stated otherwise). Data calculations were performed using Prism 4.0 (GraphPad Software Inc. San Diego, CA, USA). Statistical significance of differences of the means were determined by Student's t-test, where differences were considered statistically significant with $P<0.05$.

Results

Effects on aromatase activity in human placental microsomes

The inhibitory effects of twenty NDL-PCBs, their relevant hydroxylated and methylsulfonyl metabolites on aromatase activity were studied using human placental microsomes. Basal aromatase activity in human placenta microsomes was 1.3 ± 0.03 pmol/h/mg protein. Initial range finding experiments indicated that most of tested NDL-PCBs were inactive at 1 and 10 µM. However, PCB28 was able to inhibit aromatase activity in a concentration-dependent manner, with a calculated IC_{50} of 2.2 µM and a maximal inhibition of aromatase activity of 62.4% at 25 µM (Figure 1). The four OH-PCBs were tested in a concentration range of 6.8 nM to 27.5 µM and inhibited aromatase activity in a concentration-dependent manner. 4'OH-PCB172, 3'OH-PCB180, 3'OH-PCB182 and 5OH-PCB183 showed IC_{50} values of 25.3, 17.1, 13.57 and 12.5 µM, respectively. Further, 4'OH-PCB172, 3'OH-PCB180 and 5OH-PCB183 showed a LOEC of 3 µM and 3'OH-PCB182 showed a LOEC of 6.8 µM (Figure 2). At the highest concentration tested, 4'OH-PCB180, 3'OH-PCB182 and 5OH-PCB183 showed similar inhibition of aromatase activity of approximately 80%, whereas 4'OH-PCB172 showed an inhibition of 56.5 % ($p<0.05$).

Four environmentally relevant $MeSO_2$-PCBs, ($3MeSO_2$-PCB101, $4MeSO_2$-PCB101, $3MeSO_2$-PCB149, $4MeSO_2$-PCB149) were tested at 1.2 and 12 µM. All $MeSO_2$-PCBs tested caused a minor inhibition of aromatase activity at 12 µM, in a concentration dependent trend (Figure 3). However, this decrease was only statistically significant for and $4MeSO_2$-PCB149, when compared to control ($p<0.05$).

Effects on aromatase activity in H295R cells

The effects on aromatase activity of twenty NDL-PCBs and four relevant hydroxylated metabolites were studied using the H295R cells. Basal aromatase activity was 1.7 ± 0.5 pmol/h/mg protein. None of the tested congeners, including PCB28 which inhibited aromatase activity in microsomes (Figure. 1), were able to

induce or inhibit aromatase activity (data not shown). Further, 24h exposure of the H295R cells to dexamethasone (10 nM to 1 µM) did not change aromatase activity, indicating that in this cell line, aromatase activity is not under regulation of the glucocorticoid pathway.

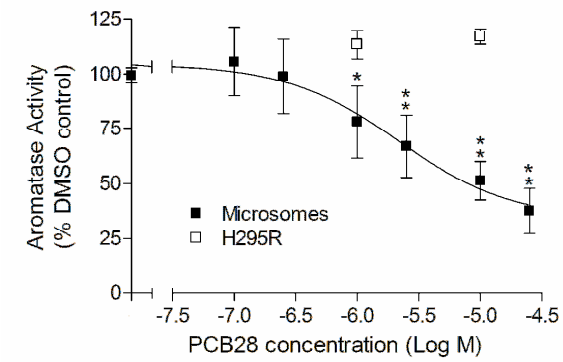

Figure 1. Concentration-dependent effect of PCB28 on aromatase activity in human placental microsomes (closed symbols) and in H295R cells at 1 and 10 µM (open symbols). Data are presented as mean ± SD (n= 8). *p < 0.01; **p < 0.001 (significantly different from vehicle control).

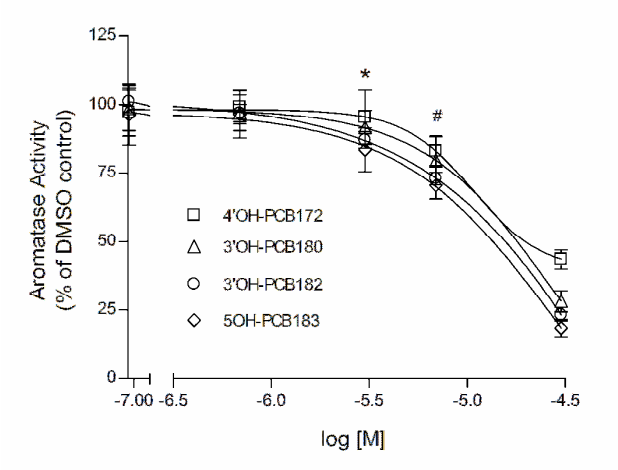

Figure 2. Concentration-dependent inhibition of aromatase activity in human placental microsomes by four OH-PCBs. Results are presented as mean ± SD (n=6). Significant difference from control vehicle are given as lowest observed effect concentration (LOEC); #p<0.02 (4'OH-PCB172, 3'OH-PCB180, 5OH-PCB183), * p<0.05 (3'OH-PCB182)

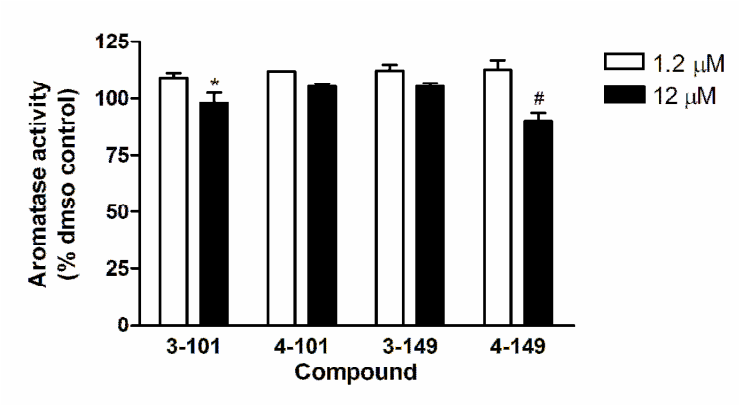

Figure 3. Effect of $3MeSO_2$-PCB101, $4MeSO_2$-PCB101, $3MeSO_2$-PCB149, $4MeSO_2$-PCB149 on aromatase activity in human placental microsomes. Data are represented as mean ± SD (n=3). * Significantly different from 1.2 μM ($p < 0.05$); # significantly different from vehicle control $p < 0.05$.

Effects on human glucocorticoid receptor activation

$MeSO_2$-PCBs have been suggested to interfere with the glucocorticoid signaling pathway (Heneweer *et al.* 2005; Johansson *et al.* 1998a), however little is known about the effects of the parent compounds and OH-PCBs. The (anti)glucocorticoid effects of a selection of PCBs and their relevant OH- and $MeSO_2$-metabolites were studied using a recently developed yeast bioassay expressing yeast enhanced green fluorescence protein and human glucocorticoid receptor (GR). Cells were initially exposed to two known GR agonists, budesonide and dexamethasone, which were able to activate the GR with an EC_{50} of 5 μM and 60 μM, respectively (Figure 4). Budesonide and dexamethasone showed concentration-dependent responses with good curve fit (r^2 of 0.935 and 0.932, respectively). The difference in glucocorticoid receptor activation potency of budesonide and dexamethasone seen in our system has also been described for mammalian cell lines (Chivers *et al.* 2004; Grossmann *et al.* 2004).

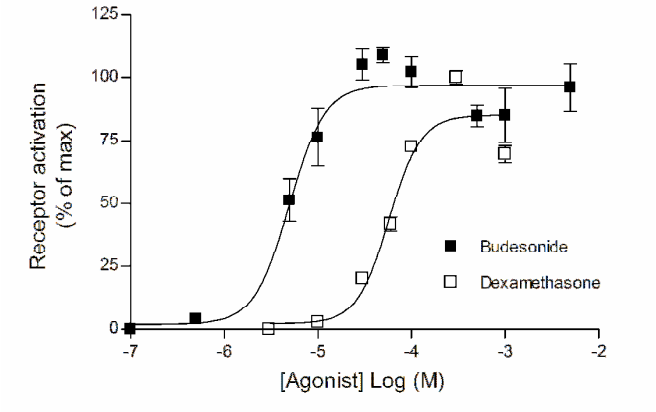

Figure 4. Concentration-response curves of two known GR agonists budesonide (closed symbols) and dexamethasone (open symbols) in the yeast glucocorticoid bioassay. Values are represented as a mean ± SD (n=6).

Of the 20 PCBs tested, most were not able to activate the GR, however, PCB52, PCB101, PCB118 and PCB153 showed a concentration dependent activation of the receptor. At the highest concentration tested (100 µM) PCB52, PCB101 and PCB118 showed a maximum activation of the receptor of 6.0, 7.2 and 7.1 % respectively, compared with the maximum activation induced by Budesonide (Figure 5A). PCB153 displayed the highest activation of the receptor at 1 µM (6.4%). However, at a concentration of 100 µM GR activation was reduced due to cytotoxicity (cf. figure 5A and 5B). PCB101 also caused a concentration-dependent decrease in yeast viability, with a maximum decrease of 49.5% compared to control at 100 µM (Figure 5B), suggesting that per living cell the GR activation is very high. PCB52 and PCB118 were not cytotoxic up to the highest concentration tested (100 µM).

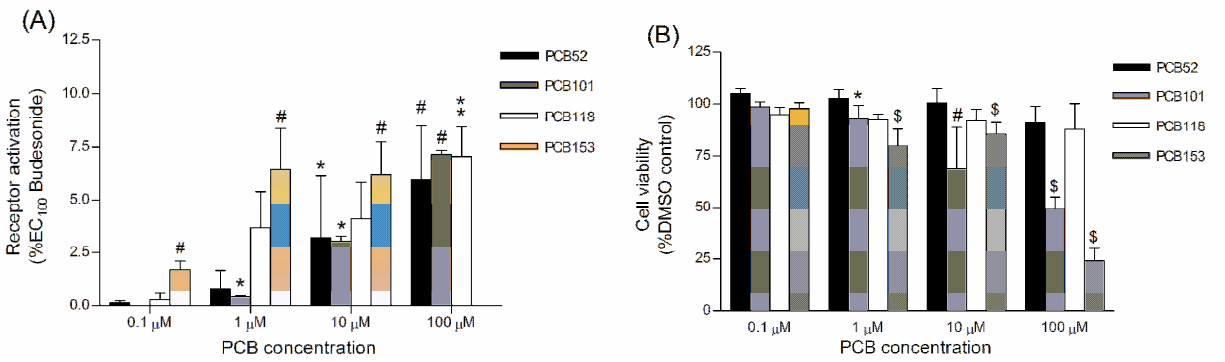

Figure 5. Effects of PCBs on **(A)** GR activation and **(B)** cell viability in the yeast glucocorticoid bioassay. The graphs depicts the PCBs that significantly showed glucocorticoid agonistic properties and the associated cell viability. Values are represented as mean ± SD (n=6) $*p < 0.05$; $**p < 0.02$; $^{\#}p < 0.005$;. $^{\$}p < 0.0005$.

The anti-glucocorticoid properties of PCBs were studied by co-exposing the cells to the PCBs and EC_{50} concentration of budesonide (5 µM). PCB19, PCB28, PCB47, PCB51, PCB52, PCB53, PCB95, PCB100, PCB101, PCB118 and PCB153 were able to inhibit the Budesonide-induced activation of the GR in a concentration-dependent manner (Table 2). However, at the highest concentration tested (50 µM), most of the PCBs were slightly, yet statistically significantly cytotoxic to the yeast cells. At 50 µM, PCB101 and PCB153 were the most cytotoxic PCBs to the yeast cells as cell viability was reduced to 37.1% and 27.9% compared to control, respectively.

Table 2. Effects of NDL-PCBs on EC_{50} concentration budesonide-induced GR activation and cell viability. Of the 20 tested NDL-PCBs, at 0.5 and 50 µM, the table only shows the congeners that significantly decreased GR activation (p<0.05) (compared to control, EC_{50} budesonide). Cell viability is only shown when significantly different from control (p<0.05). N.E. (No effect) (n=3)

	PCB concentration (0,5 µM)		PCB concentration (50 µM)	
	GR activation	Cell viability	GR activation	Cell viability
PCB19	85,0 ± 10,8%	N.E.	63,4 ± 2,4%	N.E.
PCB28	69,3 ± 11,4%	N.E.	39,4 ± 13,4%	91,3 ± 3,8%
PCB47	97,0 ± 15,5%	N.E.	48,3 ± 4,2%	91,2 ± 0,6%
PCB51	85,7 ± 1,2%	N.E.	66,9 ± 9,8%	N.E.
PCB52	88,5 ± 5,3 %	N.E.	68,5 ± 2,0%	88,4 ± 4,4%
PCB53	86,7 ± 3,5%	N.E.	69,8 ± 4,1%	95,1 ± 1,7
PCB95	84,2 ± 1,0%	N.E.	65,5 ± 7,0%	93,6 ± 1,7%
PCB100	109,9 ± 7,9%	N.E.	79,7 ± 4,3%	N.E.
PCB101	108,8 ± 8,5%	97,6 ± 1,8%	29,1 ± 11,7%	37,1 ± 11,5%
PCB118	93,3 ± 4,0%	86,7 ± 3,8	70,0 ± 2,1%	65,6 ± 15,0%
PCB153	121,5 ± 18,1%	83,2 ± 6,3%	38,1 ± 16,1%	27,9 ± 10,3%

Yeast cells were exposed to OH-PCBs for 24 hours in a concentration range of 6.9 nM to 6.9 µM. At these conditions none of the OH-PCBs was able to activate the GR (data not shown). Anti-glucocorticoid properties of OH-PCBs were tested by co-exposing the cells with EC_{50} concentration of dexamethasone (60 µM). 4'OH-PCB172, 3'OH-PCB180, 3'OH-PCB182 and 5OH-PCB183 reduced dexamethasone-induced receptor activation in a concentration-dependent manner and with IC_{50} values of 5.3, 0.2, 0.9 and 0.5 µM, respectively (Figure 6A). At the highest concentration tested, 4'OH-PCB172, 3'OH-PCB180, 3'OH-PCB182 and 5OH-PCB183 reduced the dexamethasone-induced activation of the receptor to 17.3, 21.2 and 20.8%, respectively. 5OH-PCB183 completely abolished GR activation by Dexamethasone. Further, none of congeners displayed any cytotoxicity up to the highest tested concentration (6.8 µM).

None of the $MeSO_2$-PCBs were able to activate the GR in the yeast bioassay up to 30 µM. However, when yeast cells were co-exposed to $MeSO_2$-PCBs and dexamethasone, at the highest concentration tested (30 µM), 3$MeSO_2$-PCB101 and 4$MeSO_2$-PCB149 decreased the dexamethasone-induced GR activation to 28.6 and

22.2%, respectively (Figure 6B). However, this effect was at least partly attributed to cytotoxicity, since at the referred concentration cell viability was reduced to 77.8% and 62.1 % by 3MeSO$_2$-PCB101 and 4MeSO$_2$-PCB149, respectively.

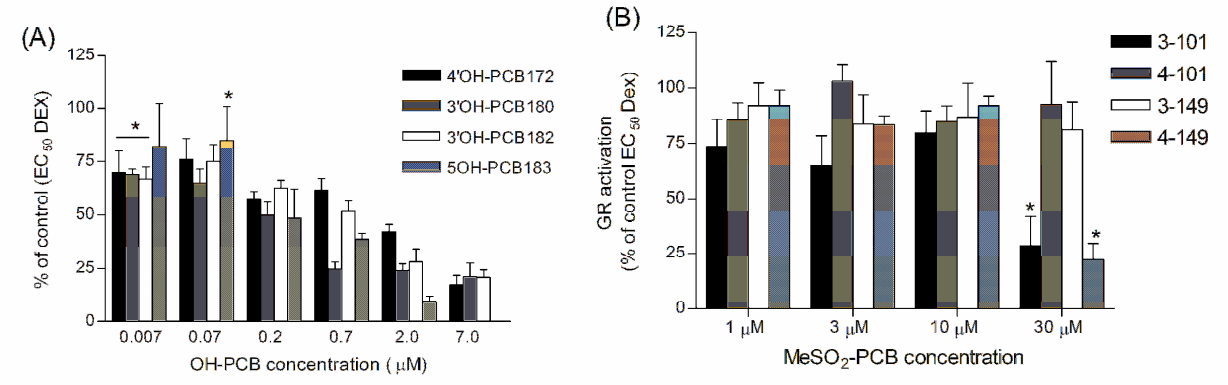

Figure 6. Anti-glucocorticoid properties of **(A)** OH-PCBs and **(B)** MeSO$_2$-PCBs in the yeast glucocorticoid bioassay in combination with EC$_{50}$ (60 μM) dexamethasone (DEX). Data are represented as mean ± SD (n=6). Significantly different from vehicle control-treated yeast cells (EC$_{50}$ DEX) are given as LOEC *p < 0.05

Discussion

PCBs are widespread environmental pollutants that can (bio)accumulate in the food chain. They can also be found in human samples, such as blood serum, cord blood and breast milk. For risk assessment, most studies have focused on the effects of the parent compounds. However, biotransformation of NDL-PCBs into their OH- and MeSO$_2$- metabolites is of great relevance since it results in systemic exposure not only to the parent compounds, but also to biologically active metabolites.

OH-metabolites can be formed after insertion of OH-group in one the phenyl rings via arene oxide formation (followed by a 1,2 shift or a NIH shift) or via direct insertion of the hydroxyl group (for review see Letcher *et al.* 2000). Therefore specific OH-PCBs can be formed from different parent PCBs. In different studies it has been observed that 4'OH-CB172, 4OH-PCB107, 3OH-PCB153, 4OH-PCB146, 3'OH-PCB138 and 4OH-PCB187 represent the majority of OH-PCBs in maternal and cord blood serum with concentrations of approximately 0.7 ng/g wet weight (Park *et al.* 2008; Soechitram *et al.* 2004). These specific OH-PCBs are most likely metabolites of the environmental most common congeners such as PCB118, PCB138, PCB153, PCB170 and PCB180. OH-PCBs are more hydrophilic than their parent compounds and may be readily excreted. However, congeners with mainly a *para*-substituted OH-group with adjacent chlorine substitution, and *meta*- and *para*-substitution of the opposite phenyl ring, tend to bind stronger to serum and plasma proteins, such as the thyroid hormone transport protein, TTR, (Brouwer *et al.* 1998 Lans *et al.* 1993). This ability to bind to proteins, in particular TTR, accounts for OH-PCBs crossing the placental

barrier and can consequently be found in higher concentrations in cord than maternal blood. This results in a higher fetal exposure to this class of compounds. Further, *in vivo* studies with mice and rats and OH-PCBs showed that these metabolites can interfere with the menstrual cycle and thyroid hormone conjugation, reduce thyroid hormone levels and display anti-estrogenic properties (Kester *et al.* 2000; Meerts *et al.* 2002; Meerts *et al.* 2004; Schuur *et al.* 1999).

$MeSO_2$-PCBs are more lipophilic metabolites than OH-PCBs and levels in human adipose tissue and milk have been found up to 1.5 ng/g lipid. When comparing levels of $MeSO_2$-PCBs in human blood plasma or milk with PCB153, the predominant congener in biota, the ratios are between 0.002 to 0.03 (Noren *et al.* 1996; Weistrand *et al.* 1997). However, these ratios can increase up to 0.128 when other tissues are taken into account, as $MeSO_2$-PCBs tend to accumulate in liver and lung (Weistrand and Noren 1997). 3$MeSO_2$-PCB101 and 4$MeSO_2$-PCB149 are among the most abundant $MeSO_2$-PCB metabolites present in humans and wildlife. Yet, the tissue retention of these metabolites is strongly dependent on the route of exposure and profile of parent compounds (Weistrand and Noren 1997; Letcher *et al.* 2002).

In the present study, twenty selected NDL-PCBs were studied as well as their most relevant metabolites, allowing a more complete hazard assessment of these compounds. NDL-PCBs have been suggested to act as endocrine disrupting compounds (Kraugerud *et al.* 2010). Therefore, our present study focused on effects on aromatase activity, which is involved in many developmental and reproductive processes, and the glucocorticoid receptor that can regulate the aromatase activity (Heneweer *et al.* 2005).

Aromatase

Human placental microsomes are a valuable tool to study catalytic activity of aromatase due to its high activity present. From all NDL-PCBs tested, only PCB28 inhibited aromatase activity in human placental microsomes. However, introduction of an OH-group resulted in a higher potency to inhibit aromatase activity, as PCB180 was unable to reduce aromatase activity, contrary to their hydroxylated metabolites. Importantly, these metabolites showed LOECs in the low µM range.

As in microsomal fraction the possible induction of aromatase can not be studied, all PCBs were also tested in the human adrenocortical H295R cell line. This cell line is well characterized and shows expression of most of the key enzymes for steroidogenesis, including aromatase (Gracia *et al.* 2006; Sanderson *et al.* 2001). In the H295R cells aromatase can only be induced via the promotors I.3 and II, but none of our PCBs or their hydroxylated metabolites caused induction of this enzyme activity.

While PCB28 and OH-PCBs inhibited aromatase activity in human placental microsomes, no changes in aromatase activity in H295R cells were observed at the

same concentrations tested. The difference in complexity of the two used systems may explain the difference in effects, as bioavailability of the PCBs in the H295R cells might play a role. In addition, H295R cells express different steroidogenic pathways, to which PCBs may influence with different affinities. This was also shown by Xu *et al*. (2006) who described that four PCBs and several of their $MeSO_2$-PCBs can simultaneously affect various genes encoding for several cytochrome P450 enzymes, hydroxysteroid dehydrogenases and cholesterol biosynthesis and transport in H295R cells (Xu *et al.* 2006). Further, the lowest observed effect concentrations (LOECs) of PCB28 and OH-PCBs in our human placental microsome studies, as a result of catalytic inhibition of aromatase activity, were in the low µM range. This, together with the effects of PCBs on different enzymes in H295R, at lower concentrations (Kraugerud *et al.* 2010), may explain the difference in effects in both *in vitro* systems.

Glucocorticoid Receptor

In the yeast glucocorticoid bioassay, the parent PCBs showed both moderate agonistic and antagonistic glucocorticoid properties. OH-PCBs and $MeSO_2$-PCBs were mostly antagonists of the glucocorticoid receptor, with OH-PCBs showing LOECs on the low nM range. However, the anti-glucocorticoid properties of $MeSO_2$-PCBs in our system were largely due to cytotocity. Anti-glucocorticoid actions of $MeSO_2$-PCBs have previously been described in a reporter cell line using Chinese hamster ovary (CHO) cells expressing the glucocorticoid response element linked to the alkaline phosphatase reporter gene, (Johansson *et al.* 1998a) and in the Reuber rat hepatoma cell line (Johansson *et al.* 2005). Further, the interaction of $MeSO_2$-PCBs with the glucocorticoid pathway appears not to be restricted to the interaction with the glucocorticoid receptor. In adrenocortical Y1 mouse cells effects of these PCB metabolites were also found for CYP11B1, a key enzyme in the production of glucocorticoids (Johansson *et al.* 1998a). The position of the $MeSO_2$ group apparently determines the ability of the metabolite to interact with the glucocorticoid receptor. This was also described by (Johansson *et al.* 2005), who showed that 4- substituted $MeSO_2$-PCBs had a stronger inhibition of the dexamethasone-induced tyrosine aminotransferase activity in rat hepatoma H4IIE-C3 cells, when compared to the 3-substituted congeners.

Glucocorticoids are able to modulate aromatase activity via P I.4. Further, the balanced production of glucocorticoids is important in the maturation of fetal organs and is involved in the proper fetal maturation and development (Ishimoto and Jaffe, 2010). Therefore the glucocorticoid pathway, regulated by glucocorticoids is an apparent target point in the potential endocrine disruptive potential of PCBs.

Interaction GR-aromatase

Previous results from our lab have identified $MeSO_2$-PCBs as inhibitors of aromatase activity in human primary mammary fibroblasts (Heneweer *et al.* 2005). Furthermore, Xu *et al.(*Xu *et al.* 2006*)*, showed that co-exposure of these metabolites and its parent compounds can also downregulate aromatase gene expression.

In the present study, we did not observe any effects of PCBs or OH-PCBs on aromatase activity in H295R cells. This is most likely due to the promoter-specific regulation of aromatase. In H295R cells aromatase expression is mainly regulated through promoter I.3/II that is upregulated by prostaglandin E2, cAMP and phorbol esters. In contrast, glucocorticoids and GR agonists are expected to act on aromatase via promoter I.4. Since this promoter is not present in H295R cells, a possible effect of PCBs and their metabolites to act via the GR on aromatase activity can not be determined in this model. However, we did observe inhibition of aromatase activity by PCBs, OH-PCBs and $MeSO_2$-PCBs in human placenta microsomes. This indicates that these compounds also modulate aromatase via direct interaction with the enzyme.

The presence of aromatase in different tissues reflects its importance on local and systemic hormonal balance. Further, perinatal exposure to PCBs resulted in modulation of aromatase, which was concomitant to the modulator effects of these compounds in developmental and reproductive end-points (Cocchi 2009, Colciago 2009). This indicates the importance of aromatase as a target point for PCBs and their metabolites.

Risk assessment

To our knowledge, this is the first study reporting direct effects of environmentally relevant NDL-PCBs, OH-PCBs and $MeSO_2$-PCBs with aromatase activity and glucocorticoid receptor. Even though metabolites are not yet taken into account in risk assessment, we observed that some PCB metabolites are more potent in interacting with aromatase activity or GR, and show LOECs in the nM range. This phenomenon was also found for polybrominated diphenylethers (PBDEs), a class of environmental pollutants structurally similar to NDL-PCBs. For PBDEs introduction of an OH-group also resulted in increased potency to inhibit aromatase activity (Canton *et al.* 2005). Yet, the effects seen in our in vitro systems are in the ppm-range, where PCBs and their metabolites in human samples are commonly found at ppb levels. For individual PCB metabolites our experiment may indicate several orders of magnitude difference in the margin of exposure (MOE) with the human situation. However, despite this apparent acceptable MOE for these PCB metabolites, it should be noticed that additivity may apply and effects of PCBs, OH-PCBs and $MeSO_2$-PCBs may be exacerbated in vivo. Therefore, their effects on aromatase and glucocorticoid

receptor can play an important role in the endocrine disruptive potential observed for these compounds.

Acknowledgments

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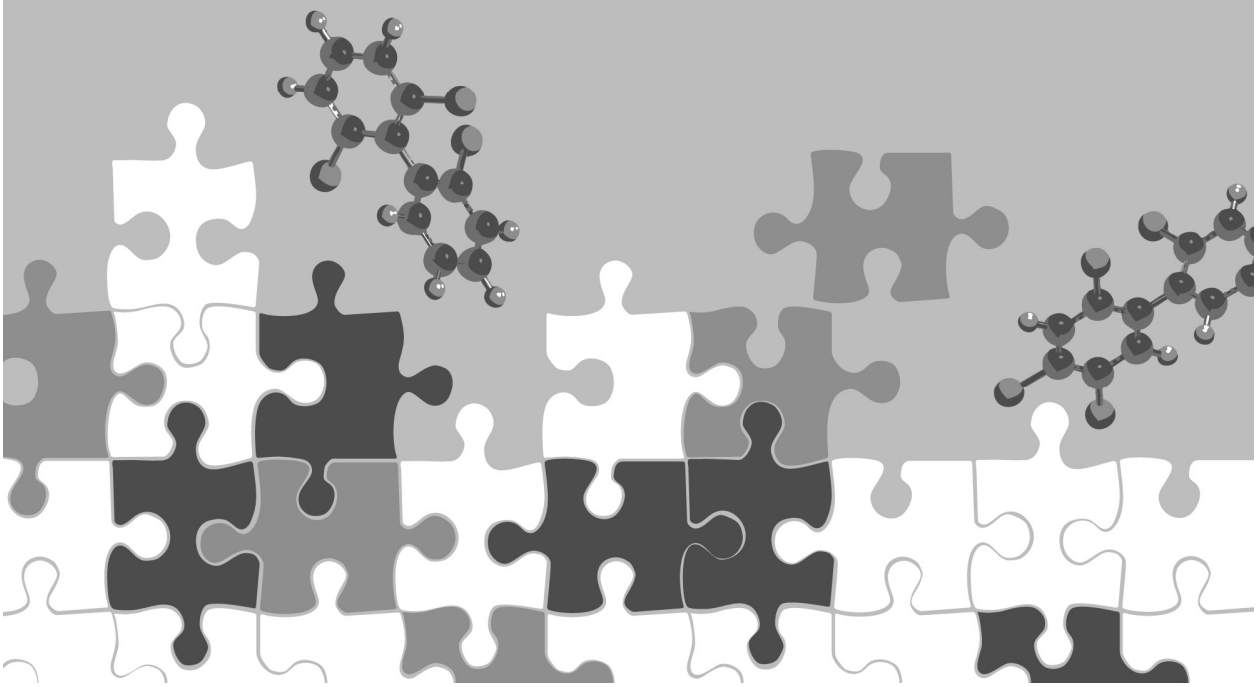
Chapter 6

Summarizing Discussion



1. Neurotoxic potential of NDL-PCBs (*in vitro*)

The ubiquity of non-dioxin-like polychlorinated biphenyls (NDL-PCBs) in the environment and human samples, together with their toxicological profile, raises concern. *In vivo* evidence demonstrated that exposure to (NDL) PCBs induces neurobehavioral effects, such as changes in motor activity, learning, memory, and attention (Eriksson and Fredriksson, 1996, Piedrafita *et al.*, 2008b, Piedrafita *et al.*, 2008a, Eriksson *et al.*, 2006b, Holene *et al.*, 1998). These effects are presumably associated with changes in the glutamatergic and dopaminergic neurotransmitter system. Alternatively, PCB exposure affects the GABA-ergic system. This is the main inhibitory neurotransmitter system in the adult mammalian central nervous system involved in motor activity as well as learning and memory (for review see (Mohler, 2007)Despite reported presynaptic effects of NDL-PCBs, information on the effects of NDL-PCBs on postsynaptic receptors was missing at the start of this doctoral research.

Chapters 2 and **3** show a novel mode of action for NDL-PCBs: activation and/or potentiation of the postsynaptic $GABA_A$ receptor. This effect was exclusive for lower-chlorinated NDL-PCBs with 1-3 chlorines in the *ortho*-position and an equal number of meta-substitutions (0-1) on both phenyl rings. Further, these should not have adjacent *para*- and *meta*-substitution on the same phenyl ring. The structure activity relationship (SAR) established in the present studies is not in complete accordance with other previously established SARs for ryanodine receptors (RyR) activation or neurotransmitter uptake by synaptic vesicles and synaptosomes. This stresses the idea that the neurotoxic profile of NDL-PCBs depends on the end-point considered.

Further, our *in vitro* system shows effects at concentrations comparable with, or even below, those previously reported for effects on presynaptic processes. The Lowest Observed Effect Concentration (LOEC) for activation and potentiation of the $GABA_A$ receptor by the most potent congener, PCB47, amounted to 0.1 and 0.01 µM, respectively. The plasma levels of individual lower-chlorinated NDL-PCBs, such as PCB28 and PCB47, after exposure to contaminated indoor air amount to 0.02-0.03 nM, respectively (Liebl *et al.*, 2004).

Despite the magnitude of difference between the effects levels seen in this *in vitro* test and blood plasma levels, we show in **chapters 2** and **3** that binary mixtures of lower-chlorinated NDL-PCBs were able to potentiate the $GABA_A$ receptor in an additive manner. However, higher-chlorinated NDL-PCBs bind to the $GABA_A$ receptor apparently in a competitive manner, thereby inhibiting the potentiating effect of the lower-chlorinated congeners. **Chapter 2** and **3** thus stress that the effect of a mixture on the $GABA_A$ receptor depends on the mixture composition.

Moreover, it was recently observed that the lower-chlorinated NDL-PCB47 was able to inhibit the excitatory acetylcholine receptor (AChR; (Hendriks *et al.*,

2010)). Whether the SAR established in this thesis for the effects of NDL-PCBs on $GABA_A$ receptor can be extended to AChRs is still unclear. Nonetheless, these opposite (and additive) effects, i.e., potentiation of the inhibitory $GABA_A$ receptor and inhibition of the excitatory ACh receptor, likely augment the effects observed *in vivo*.

$GABA_A$ receptors contain several binding sites for different classes of (ant-) agonists, such as anxyolitics, ethanol and neurosteroids. *In vivo* and *in vitro* results have shown that prolonged use of xenobiotics, e.g., anaesthetics and anxiolytic drugs, can disrupt the function of $GABA_A$ receptor and eventually lead to cell death (Ikonomidou, 2010, Olney *et al.*, 2000). Mainly during neurodevelopment, the administration of anaesthetics, alcohol (etc) in humans, have resulted in attention deficit and lower IQ scores (Ikonomidou, 2010, Henry *et al.*, 2007). Therefore, the ability of some NDL-PCBs to modulate the $GABA_A$ receptor may be translated into similar effects as other agonists of this receptor.

The combined findings thus support the idea that the $GABA_A$ receptor should be taken into consideration when assessing the neurotoxic potential of NDL-PCBs. Although not dealt with in this thesis, there is evidence that the $GABA_A$ receptor is also present in gonads and adrenal glands in rats (Akinci and Schofield, 1999). This indicates that the acute effects seen in our *in vitro* system may also affect the endocrine system and are not specific for the central nervous system (CNS) only.

2. Endocrine effects

In **chapter 4**, rats perinatally exposed to NDL-PCB52 showed an induced aromatase activity on adult female adrenals and ovaries. This indicates that the effects are sex dependent, as aromatase activity measured in male adrenals and testis was apparently not influenced by perinatal exposure. Sex-related differences in PCB effects have also been suggested in a human study on onset of puberty, which was hastened in females and delayed in males (Dickerson *et al.*, 2011b). The higher sensitivity of females to the disruptive effects of NDL-PCBs may be explained by a lower neonatal steroid hormone levels, when compared to males (Bakker and Brock, 2010). Other studies have also shown the higher sensitivity of females to PCB effects. Dickerson *et al.*, (2011a) have shown that female rats were more sensitive to perinatal exposure to reconstituted mixtures of NDL-PCBs (PCB138, PCB153 and PCB180), up regulating aromatase and ERα gene expression in female, but not male brain.

In addition, a lower metabolic rate in females may account for a longer systemic exposure, which could underlie the observed sex-difference in toxicity. Rats exposed to the hexabromocyclododecane (HBCD), a class of brominated flame retardants (BFRs), revealed that, compared to male rats, females showed a higher

hepatic levels of the parent compound and a down regulation of hepatic genes involved in conjugation processes. (van der Ven *et al.*, 2009, Canton *et al.*, 2008).

In addition, studies with BDE47, a BFR structurally similar to NDL-PCBs, showed that compared to male, female mice presented slower congener elimination and metabolite formation (Chen *et al.*, 2006, Staskal *et al.*, 2006).

In **chapter 4**, the effects we observed were also congener dependent. Animals exposed to NDL-PCB180 did not show any apparent effect on aromatase activity, in contrast with NDL-PCB52. In addition, gene expression of androgen receptor (AR) in adult brain was reduced in animals perinatally exposed to NDL-PCB180, but not NDL-PCB52. Recently, Boix *et al.*, (2010) have shown that after perinatal exposure, accumulation of these two congeners in rat brain is different; contrary to what happened to PCB180, PCB52 could no longer be detected. This again suggests that a longer systemic exposure to NDL-PCBs may account for the observed toxic effect.

Several studies investigated the endocrine effects of perinatal exposure to PCBs (Colciago *et al.*, 2009, Hany *et al.*, 1999, Kaya *et al.*, 2002). However, most of the studies also include DL-PCBs in the mixture, which are also known to have endocrine modulating effects, *in vivo* (Yamamoto *et al.*, 2005). These may have influenced the attribution of the effects to *ortho*-congeners. Nevertheless, the reported effects were permanent and detected even at a later age. In line with our findings, this indicates that PCB exposure during a critical window of development may have later adverse effects in neuroendocrine system.

During fetal development, hormonal production by fetal and maternal glands and placenta, are in direct interaction and dynamic, forming the mother-placental-fetal unit. This accounts for an estrogen-synthesizing system, which plays a key role in the maintenance of pregnancy, fetal maturation and development and, in some species, the initiation of parturition (Ishimoto and Jaffe, 2010). Placental aromatase is directly involved in the ratio androgens/estrogens that the foetus is exposed to. Human placenta does not produce estrogens *de novo*, from cholesterol due to its incomplete steroidogenic pathways. Therefore it is dependent on the maternal and foetal supply of 19-carbon steroid hormones (such as androgens and Dehydroepiandrosterone (DHEA)), which are ultimately substrates for aromatase for the production of estrogens. Both PCBs and their hydroxylated metabolites have been shown to cross the placental barrier resulting in significant systemic exposure of the foetus (Correia Carreira *et al.*, 2011, Park *et al.*, 2008). Taken together, this suggests that some endocrine effects of NDL-PCBs can be mediated through interaction with the aromatase enzyme.

Chapter 5 takes a more mechanistic approach on the endocrine effects of NDL-PCBs by studying the effects on aromatase activity and the glucocorticoid receptor. PCB28, four hydroxyl-PCBs (OH-PCBs) and four methylsulfonyl-PCBs

($MeSO_2$-PCBs) were identified as catalytic inhibitors of aromatase activity in the human placenta. In view of these results it should be recognized that aromatase expression is highly tissue and promoter specific regulated. In human adrenal corticocarcinoma (H295R) cells, aromatase is under control by the promoters I.3 and II, which are regulated by prostaglandin E2 (PGE2), cyclic adenosine monophosphate (cAMP) and Phorbol esters. In other tissues, e.g. human adipose, aromatase is regulated by another promoter region (I.4) that is regulated by glucocorticoids. Since this promoter region is not present in H295R, a possible effect of PCBs and their metabolites to act via the glucocorticoid receptor (GR) pathway on aromatase could not be determined using this model. Therefore, a recently developed yeast bioassay, described in **chapter 5**, was used to study NDL-PCBs and their OH- and $MeSO_2$-PCBs for their (anti)-glucocorticoids properties. Most of the tested NDL-PCBs were (anti-) glucocorticoids. Interestingly, when testing four OH-metabolites of the common, but inactive NDL-PCB180, all metabolites showed anti-glucocorticoid properties in contrast with the parent compound. Further, some phase II metabolites of PCBs, $MeSO_2$-PCBs, also showed anti-glucocorticoid properties, dependent on the position of the $MeSO_2$-group in the phenyl group. Thus, hydroxy- and methylsulfonyl-metabolites of NDL-PCBs were stronger (anti-)glucocorticoids and aromatase inhibitors, than their parent compounds. This bio-activation towards a specific end-point is a shared characteristic with polybrominated diphenylethers (PBDEs) that are structurally similar to NDL-PCBs. For these PBDEs, introduction of an OH-group also resulted in increased potency to inhibit aromatase activity, decreasing cell viability, generation of reactive oxygen species and TTR binding. In contrast, the potency of PBDEs was reduced by phase II metabolism, as methyl-PBDEs showed a lower/reduced effects on the same end-points described above (for review see (Dingemans *et al.*, 2011)).

The novel finding that OH-PCBs can interact with the GR strengthens the concept that endocrine disruption can arise via various modes of action. This highlights the importance of looking at different endocrine end-points (e.g. enzymes and receptors), in which different organs interact and can influence each other Interestingly, only PCB28 was able to inhibit aromatase activity, whereas most of the tested NDL-PCBs showed (anti) glucocorticoid properties. Moreover as aromatase inhibitors OH-PCBs showed LOECs in the low µM range, whereas as antiglucocorticoids they showed LOECs in the low nM range. This indicates a potential more sensitive mechanism of action for the latter interaction at environmental relevant concentrations.

3. Risk assessment

3.1. Exposure to NDL-PCBs

For the general population, food is the main route of exposure to NDL-PCBs. In 2005 the European Food Safety Authority (EFSA) has identified six common NDL-PCBs, which alone account for more than 50% of all NDL-PCBs present in food and feed. NDL-PCB153 is commonly used as the reference congener due to its high contribution in biota and abiota.

Inhalation exposure to NDL-PCBs occurs mainly via indoor air. The higher indoor exposure levels of, specifically, lower-chlorinated NDL-PCBs is often reflected in the blood levels, where the ratio of these congeners to higher-chlorinated ones is significantly higher than in other matrices (Liebl *et al.*, 2004, Gabrio *et al.*, 2000, Harrad *et al.*, 2009). Importantly, similar to BFRs, the abundant presence of NDL-PCBs in house dust has raised concern on this route of exposure, mainly for neonates, toddlers and children. During this time period, there is an ongoing development and maturation of organs, when elimination systems are not yet complete, i.e. metabolizing enzymes and renal function. This taken together with hand-to-mouth behaviour, accounts for a higher exposure per body weight (bw) of neonates, children and toddlers, when compared to adults. (Harrad *et al.*, 2010, Schwenk *et al.*, 2003). House dust and compounds adsorbed to house dust may enter the human body by inhalation or ingestion. NDL-PCBs can adsorb to particles with different affinities depending on the chlorination pattern of the congener. Dust contributes proportionally more to exposure of higher chlorinated NDL-PCBs, as opposed to the lower-chlorinated congeners that are more abundant in air.

In epidemiological and *in vivo* experimental studies two different time periods of PCB exposure have been identified of high importance: perinatal and chronic exposure (Jacobson *et al.*, 1990, Alcock *et al.*, 2000).

- Perinatal exposure

Organ development in the fetus, neonate and young children is highly sensitive to changes in hormonal environment. In this respect, most knowledge on the effect of the steroids on brain development comes from rodent models. In rodents the “brain growth spurt” starts in the first 3-4 weeks of gestation and it is also during this period that brain sexual differentiation occurs. Female rodent brain develops in the relative absence of steroid hormones, whereas the male brain is exposed to higher levels of testosterone and estradiol. In humans the “brain growth spurt” starts in the third trimester of pregnancy and continues throughout the first five years of life. This is a period in which the brain undergoes several developmental processes, such as neurogenesis, proliferation, migration and differentiation. These

early age processes are of critical importance for (the attainment and maintenance of) adult reproductive functions. Insults during this period may have effects that persist in adulthood.

Therefore, the hypothalamus-pituitary-gonads (HPG) and hypothalamus-pituitary-adrenal (HPA) axis have received much attention. The organizational structure and function of the brain, and in particular sexual dimorphic regions, is very dependent on the circulating levels of hormones produced by the gonads and the adrenals, but also on (sex) hormones produced *de novo* in the brain.

Figure 1. shows a schematic overview of the hormonal interaction between the different organs in the HPA and HPG axis.

During gestation the placenta is not an effective barrier to protect the foetus from xenobiotic insults (Correia Carreira *et al.*, 2011, Park *et al.*, 2008). Further, it is also during this period that the mother, the placenta and the foetus form the so-called mother-placental-foetus unit. The ability of compounds to cross the placenta is among others related to their ability to bind to proteins. OH-PCBs have been found to bind strongly to the transthyretin (TTR), the thyroid hormone transporting protein. This protein passes the placenta and blood-brain barrier, possibly being responsible for effects on e.g. neurodevelopment. Further, adrenal glucocorticoids are involved in the duration of pregnancy, but also in the maturation of foetal organs and foetal growth. Changes in glucocorticoid levels during foetal development (Gonzales *et al.*, 1990, Bolt *et al.*, 2001) may have consequences throughout life: new-borns with lower weight at birth have usually higher cortisol levels throughout life.

- Lifetime or cumulative exposure

As described above, perinatal exposure to pollutants, such as PCBs, can have persistent effects in to adulthood. Chronic exposure to PCBs is an exposure over the (a long part of the) lifetime of the individual, stretching from prenatal, postnatal, childhood to adult exposure. This often results in a difficulty to discern time exposure effects. In adult age, organs have already been formed and reorganizational effects are not expected. However, PCBs can still interact with receptors/enzyme directly and or at expression level, activating cascades of events with in the cells of those organs.

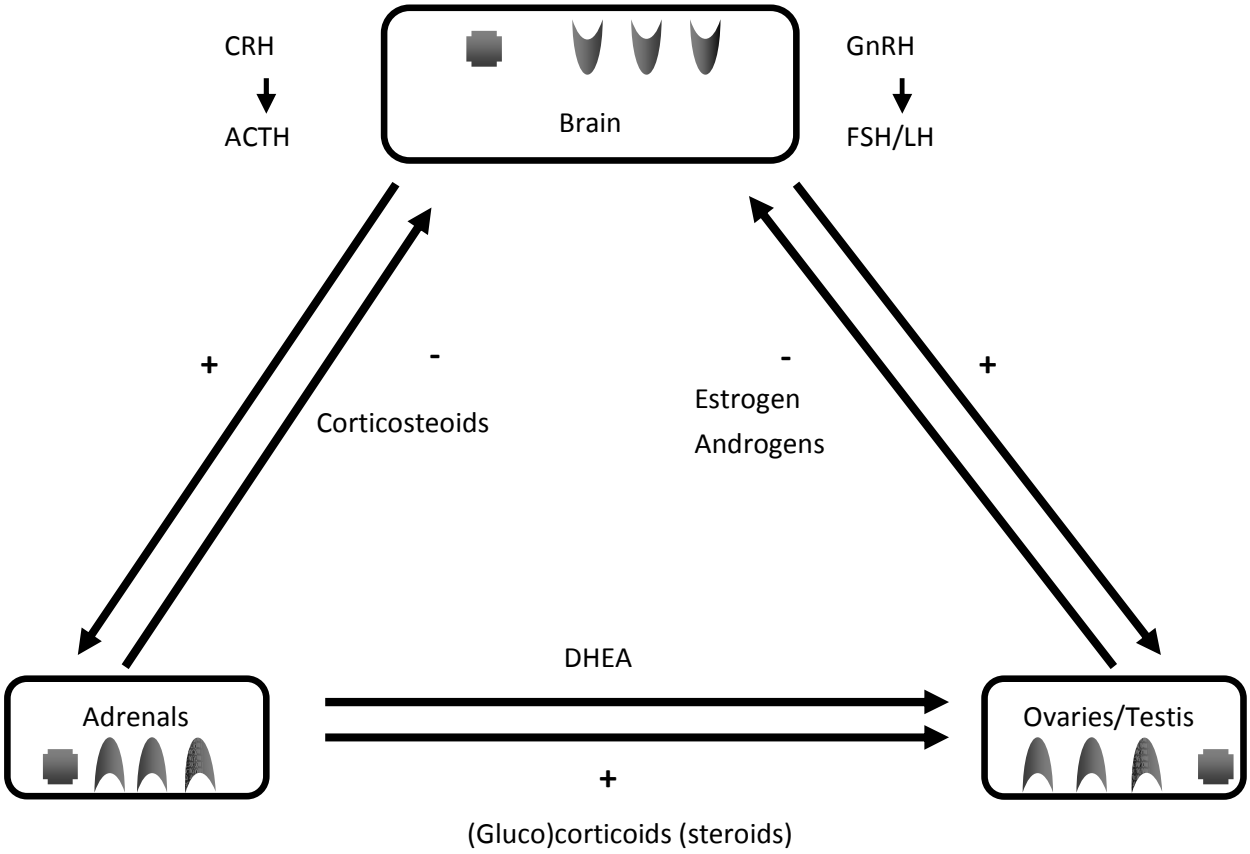


Figure 1. Schematic overview of hormonal interaction between the HPA and HPG axis. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; CRF, corticotropin releasing factor; ACTH, adrenocorticotropic hormone; DHEA, dehydroepiandrosterone; + positive feedback; - negative feedback. steroid receptor: estrogen receptor, androgen receptor or glucocorticoid receptor; aromatase enzyme.

3.2. PCB metabolism

Most OH-PCBs are readily excreted via faeces and urine, but some of them can still be found in human blood serum (Park *et al.*, 2008, Soechitram *et al.*, 2004). These compounds also have a stronger affinity for specific transporter proteins, such as the TTR, enabling these metabolites to cross the placental barrier more easily. Concentrations of certain OH-PCB congeners in cord serum are higher than in maternal serum. The interaction of (OH-)PCBs with the TH homeostasis, via TTR and thyroid hormone receptor binding (Kawano *et al.*, 2005, Sandau *et al.*, 2002), has been pointed out regularly as a possible cause and mechanism underlying the observed neurodevelopmental effects of PCBs (Patandin *et al.*, 1998). This is important, because these OH-PCBs have been detected in foetus and can either pass the placental membrane or be formed directly in the foetus.

$MeSO_2$-PCBs are formed via phase II enzymes. The methylsulfonyl group is attached to the biphenyl backbone and responsible for a decrease in hydrophilicity compared to phase I metabolites (OH-PCBs). Due to binding to specific proteins, these $MeSO_2$-metabolites can accumulate in lung and liver, but also in human milk. There has been a long and ongoing discussion to which extent neonates and toddlers should be considered as a separate target group in risk assessment. The high daily exposure to NDL-PCBs via human milk, often two orders of magnitude higher than adults, certainly warrants this differentiation (European Food Safety Authority (EFSA), 2005). The tissue specific accumulation of $MeSO_2$-PCBs in human milk certainly adds up to the exposure of the neonate compared to humans.

As a result of background exposure, levels of individual PCBs and their metabolites in human tissues and serum are usually in the sub to low nM range (Park *et al.*, 2008, Petrik *et al.*, 2006, Hovander *et al.*, 2006). In the different in vitro systems, used in this doctoral research most effects are seen at µM level.

Despite this order of magnitude difference, it should be noticed that additive effects resulting from co-exposure of PCBs, OH-PCBs, $MeSO_2$-PCBs and also other pollutants may be expected (Hendriks *et al.*, 2010, Gao *et al.*, 2009, He *et al.*, 2009b). Such results are likely to exist also in the *in* vivo situation. Moreover, in **chapter 5** we describe that OH-PCBs show antiglucocorticoid properties at nM range. Therefore there appears to be a good scientific rationale to include PCB metabolites also in the human risk assessment, especially for breast fed infants.

3.3. Effect of mixtures

Occurrence and environmental exposure to PCBs is concomitant to other pollutants, such as methyl mercury (MeHg) and brominated flame retardants, which is reflected in human body burden. However, there is still a serious lack of knowledge on the mechanistic interaction of these different classes of pollutants on the different systems that that these may affect.

Pre- and postnatal exposure to MeHg has been related to cognitive and motoric alterations in children (Myers and Davidson, 1998, Nakai and Satoh, 2002, Bland and Rand, 2006). In addition, toxicity studies with mixtures of PCBs and MeHg revealed interaction between these contaminants depending on the *in vitro* system and endpoints used. While MeHg and PCBs show synergism in decreasing the dopamine content in slices from rat striatum, antagonistic effects on intracellular calcium in cerebellar neurons in culture were also observed (Bemis and Seegal, 2000, Coccini *et al.*, 2006) showed that the increase in density of muscarinic receptors in cerebellum by MeHg is impaired if co-applied with PCBs. However, the mechanisms behind these non additive mixture effects are not completely understood. In this thesis, (**Chapters 2** and **3**) it is reported that binary mixtures of lower-chlorinated

PCBs were able to potentiate the $GABA_A$ receptor in an apparently additive manner. However, this additive effect was reduced when the lower-chlorinated PCB47 was co-applied with the higher-chlorinated NDL-PCBs, PCB153 or PCB180. These results clearly indicate that the composition of the PCB mixture and relative concentrations of each congener determine the net effect on the $GABA_A$ receptor. As said earlier, human exposure to environmental pollutants is not restricted to PCBs and several *in vivo* and *in vitro* studies reported that co-exposure of PCBs and PBDEs can also result in additive neurotoxicity (Hendriks *et al.*, 2010,Gao *et al.*, 2009,He *et al.*, 2009b,He *et al.*, 2009a). In particular, *in vivo* experiments using a prenatal exposure scenario have shown that behavioural effects of NDL-PCBs can be worsened by co-exposure with the environmental important PBDE99 (Eriksson *et al.*, 2006a). These findings have been supported recently with *in vitro* studies in which co-exposure of a lower chlorinated NDL-PCB with a hydroxylated PBDE metabolite PCB47 can potentiate the Ach receptor (Hendriks *et al.*, 2010).

3.4. Structure activity relationship of (NDL-) PCBs on different end-points

Due to the large number of NDL-PCBs, *in vivo* testing is not feasible from an economical, labour and animal welfare point of view. Therefore, computational toxicology is of high relevance to predict toxicological properties of this complex group of compounds.

In the present thesis (**chapter 2** and **3**), chemometric testing of the effects of a set of twenty NDL-PCBs and two DL-PCBs on the $GABA_A$ receptor has indicated, that the activation or potentiation of the receptor is apparently exclusive for core structure of NDL-PCBs. More precisely, NDL-congeners with more than five chlorine atoms were not able to activate or potentiate the $GABA_A$ receptor and the number of *ortho*-chlorination should be restricted to 3 or less. Moreover, both phenyl rings should have an equal number of meta-substitutions (0-1) and no adjacent *para*- and *meta*-substitutions on the same phenyl ring.

Mariussen and Fonnum, (2001) also described the potential of NDL-PCBs to inhibit vesicular and membrane neurotransmitter uptake, in contrast to DL-PCBs. More specifically, NDL-PCBs with penta- and hexachlorinated congeners with 2 to 4 chlorine atoms in *ortho*-positions were the most potent congeners in inhibiting vesicular neurotransmitter uptake, with IC_{50} values in the low µM range. The same research group (Mariussen *et al.*, 2001) has described that *ortho*-chlorinated PCBs with four to five chlorine substituents, were the most effective in inhibiting membrane neurotransmitter uptake, with again IC_{50} values in the low µM range. In contrast, the hexa- or heptachlorinated PCBs were poor or partial inhibitors of glutamate as well as GABA cellular uptake.

Other *in vitro* experiments with rat pheocromocytoma (PC12) cells have also identified NDL-PCB with *ortho*- or *ortho*- and *para*-chlorine substitution as the most

potent congeners that can decrease dopamine concentration. However, looking only at the relative numbers of chlorine substitutions was not found to be a good indicator of the congeneric potency: complete substitution of all *ortho*-positions decreased toxicity, while additional chlorine atoms in the *para*-position increased toxicity. Further, chlorine substitutions in the *meta*- positions, 3 or 5, seems to decrease the potency of *ortho*-, *para*-substituted NDL congeners (Shain *et al.*, 1991)
Pessah *et al.*, (2010) established a SAR for activation of the ryanodine receptor (RyR) by NDL-PCBs and described that *ortho*- and *meta*-chlorine substitutions on the biphenyl structure are the most important determinants. Interestingly, this RyR potency was apparently not dependent on total chlorination of the molecule.

PCBs are also found to be estrogenic in the MCF-7 (human mammary adenocarcinoma cell line) proliferation assay. Using four different congeners it was shown that the estrogenic potency was dependent on the chlorination substitution pattern, where chlorines in two to four *ortho*-positions and one *para*-position showed the highest estrogenic potency (Andersson *et al.*, 1999). Using the same *in vitro* MCF-7 proliferation assay, but with a larger set of congeners (34), DeCastro *et al.*, (2006) was able to rank the congeners in order of estrogenicity according to their chlorination pattern. The authors showed that all estrogenic PCBs were at least *ortho*-substituted with the most estrogenic congeners having an *ortho* and *para*-substitution pattern. However, the total number of chlorine atoms was not a clear determinant for estrogenicity. In a latter study, the most potent congeners showed LOECs in the low µM range. These results are in line with another study using the human mammary ductal carcinoma cell line T47D transfected with a luciferase reporter gene controlled by the estrogen responsive element (Pliskova *et al.*, 2005). In this study it was again found that lower-chlorinated NDL-PCBs were the most potent estrogenic congeners, while higher chlorinated congeners showed anti-estrogenic properties.

When reviewing the above presented results it must be concluded that neuro-endocrine effects of NDL-PCBs are governed by apparent different SARs depending on the endpoint and biological system used. As a result, it is difficult to generalize a SAR for neuro-endocrine responses of NDL-PCBs in spite of their environmental abundancy and importance for risk assessment.

4. Main conclusions

- Lower chlorinated-NDL-PCBs activate and/or potentiate the $GABA_A$ receptor as a novel mode of action.

- The established SAR for $GABA_A$ receptor is not completely similar with previous SARs identified for other important endpoints in neurotransmission.

- Binary-mixtures of lower-chlorinated NDL-PCBs potentiate the $GABA_A$ receptor in an additive manner, but higher-chlorinated NDL congeners may (partially) inhibit this potentiation. Consequently, the composition of the PCB mixture will determine the net effect on this receptor.

- Effects on the $GABA_A$ receptor add to the neurotoxic potential of NDL-PCBs.

- Perinatal exposure to NDL-PCB52 and NDL-PCB180 affect differently androgen receptor expression in rat brain, and aromatase activity in gonads and adrenals.

- Effects of perinatal exposure to NDL-PCBs on neuroendocrine system are age and sex dependent for the offspring and may persist into adulthood.

- NDL-PCBs and their metabolites OH-PCBs and $MeSO_2$-PCBs are at least *in vitro* inhibitors of aromatase and glucocorticoid receptor.

- The observed interaction of OH-PCBs with the glucocorticoid receptor is a novel mode of action.

- PCB-metabolites are stronger aromatase inhibitors than their parent compounds. When assessing the endocrine disruptive potential of NDL-PCBs, possible effects of their metabolites should also be taken into account.

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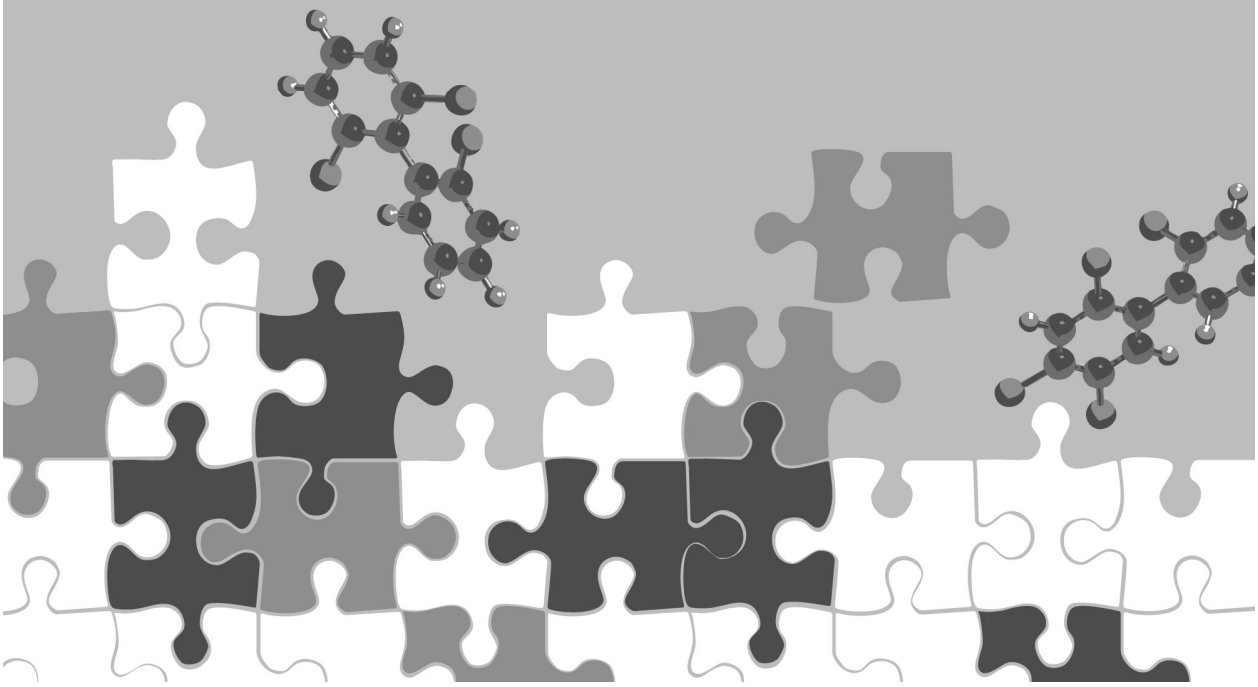
Nederlands Samenvatting



Inleiding

Polychloorbifenylen (PCBs) is een klasse van synthetisch organische stoffen bestaande uit 209 theoretische PCB congeneren, die verschillen in het aantal chlooratomen en de locatie hiervan aan elke fenyl groep. De plaats van binding van de chlooratomen aan de fenyl groepen bepaalt hoe deze zich ruimtelijk gedragen ten opzichte van elkaar. Op basis van deze eigenschap kunnen twee klassen PCBs worden onderscheiden. De dioxine-achtige PCBs (DA-PCBs) waarbij de twee fenyl groepen zich vrijwel in hetzelfde vlak bevinden en de niet-dioxine-achtige PCBs (NDA-PCBs) waarbij de twee fenyl groepen (deels) gekanteld te op zichte van elkaar staan.

Door het verbod op de productie en het commerciële gebruik van zowel DA- als NDA-PCBs zijn de concentraties in het milieu van deze persistente organische vervuilende stoffen de laatste decennia langzaam gedaald. Echter, doordat de stoffen zo persistent zijn, worden ze nog steeds teruggevonden in het milieu en in de mens.

DA- en NDA-PCBs kunnen zowel door fase I enzymen worden omgezet tot hydroxy-PCBs (OH-PCBs) als door fase II enzymen tot bijvoorbeeld methylsulfonyl-PCBs ($MeSO_2$-PCBs). Ondanks dat het merendeel van de OH-PCBs via de feces of urine uitgescheiden wordt, kunnen ze nog altijd gevonden worden in het bloed van mensen en dieren, waarbij ze vooral gebonden zijn aan eiwitten. De $MeSO_2$-PCBs hebben ten opzichte van de oorspronkelijk PCBs slechts een iets lagere vetoplosbaarheid, wat de affiniteit voor vetweefsel van deze faseII metabolieten verklaart.

Door het trage metabolisme van PCBs in mens en dier treedt bioaccumulatie en -magnificatie van zowel de DA- als NDA-PCBs op, waardoor relatief hoge concentraties gevonden worden bij soorten in de top van de voedselketen, inclusief de mens. Meer dan 90% van de NDA-PCBs die mensen binnen krijgt komt via voeding het lichaam in. Van deze NDA-PCBs zijn het vooral de PCBs met veel chlooratomen (bijv. PCB138, PCB153 en PCB180) die kwantitatief het meest voorkomen in dierlijk voedsel. PCBs met minder chlooratomen die door hun chemische structuur vluchtiger zijn (bijv. PCB28 en PCB52), kunnen via inademing resulteren in humane blootstelling.

Mensen worden hun hele leven blootgesteld aan PCBs. Dit geldt ook voor de kritische ontwikkelingsfasen van ongeboren kinderen, pasgeborenen die borstvoeding krijgen en peuters die groeispurten doormaken. Inmiddels is bekend dat PCBs (en hun metabolieten) een belangrijk effect hebben op de ontwikkeling van het zenuwstelsel, de hormoonhuishouding en de voortplanting. Om deze reden is het van groot belang dat met name de blootstelling van het (ongeboren) kind wordt meegenomen in risicobeoordeling van deze stoffen.

Het neuro-endocriene systeem omvat twee belangrijk regulerende systemen in het menselijk lichaam; het zenuw- en hormoonstelsel. Interacties tussen beide

systemen kan o.a. plaatsvinden via hormonen, waarbij vervolgens de aansturing van andere organen kan plaatsvinden. Een voorbeeld hiervan zijn de geslachtsgebonden verschillen in de hersenen, een proces dat mede onder controle staat van de hormoonhuishouding. Hoewel het brein in staat is het vrouwelijke hormoon oestradiol enzymatisch (via aromatase) te produceren, kunnen de concentraties van andere circulerende hormonen, zoals gonadotropines, ook de activiteit en werking van geslachtshormoon-enzymen en activiteit van hormoonreceptoren beïnvloeden. Veranderingen in de (geslachts)hormoonhuishouding gedurende de ontwikkeling van een kind kan mogelijk leiden tot permanente verandering in het neuro-endocriene systeem. Een dergelijk effect kan veroorzaakt worden in het voortplantingssysteem maar mogelijk ook (indirect) zorgen voor neurologische gedragseffecten.

De kans op neurologische gedragseffecten en hormoonverstoring door blootstelling aan NDA-PCBs is het grootst in de ontwikkelingsfasen van de mens. Uit epidemiologische en experimentele onderzoek komen de schadelijke effecten van PCBs op ongeboren en pasgeboren kinderen en dieren duidelijk naar voren. Deze blootstelling kan dus een negatieve invloed hebben op de ontwikkeling van het zenuwstelsel, het gedrag en het voortplantingssysteem. Zorgwekkend is hierbij ook dat dergelijke effecten mogelijk van blijvende aard zijn en dus ook op volwassenen leeftijd een rol kunnen spelen.

NDA-PCBs beïnvloeden humane $GABA_A$ receptoren

Omdat er slechts beperkt onderzoek is gedaan naar de schadelijk effecten van NDA-PCBs op het zenuwstelsel, is risicoclassificatie en -beoordeling van dit type PCBs nog steeds problematisch. Hoewel het effect van NDA-PCBs op het presynaptische deel van de communicatie tussen zenuwcellen reeds bekend is, was het effect van NDA-PCBs op de postsynaptische neurotransmitter receptoren aan het begin van dit promotieonderzoek nog onbekend. Hoofdstuk twee beschrijft dan ook de effecten van zes veel voorkomende NDA-PCBs (PCB28, PCB52, PCB101, PCB138, PCB153 en PCB180) op humane $GABA_A$ receptoren. GABA and $GABA_A$ receptoren behoren tot het GABA-erge systeem. Dit is het belangrijkste remmende neurotransmitter systeem in het volwassen centrale zenuwstelsel en speelt een belangrijke rol bij o.a. motorische activiteit, leren en geheugen. $GABA_A$ receptoren werden tot expressie gebracht in eicellen van de Afrikaanse Klauwkikker (*Xenopus laevis).* Na blootstelling aan deze PCBs werd vervolgens de activatie van de $GABA_A$ receptoren bestudeerd met de zogenaamde twee-elektroden voltage clamp techniek.

De resultaten die in dit hoofdstuk worden beschreven, laten een nieuw werkingsmechanisme voor NDA-PCBs zien. NDA-PCBs met een laag aantal chlooratomen (≤4) als PCB28 en PCB52 werken als partieel agonist op de humane $GABA_A$ receptor, terwijl PCB congeneren met meer chlooratomen (≥5) en de dioxine-

achtige PCB126 dit effect niet hebben. Het partieel agonistische effect van PCB28 en PCB52 is afhankelijk van de binding van de natuurlijke agonist GABA aan de $GABA_A$ receptor. Dit werd geconcludeerd omdat het partieel agonistische effect van deze twee PCBs alleen optreedt wanneer GABA is toegevoegd bij een effectieve concentratie van 20% (EC_{20}) en niet meer bij een EC_{80} concentratie.

Aangezien humane blootstelling aan NDA-PCBs altijd plaatsvindt door mengsels, werden ook binaire mengsels van deze PCBs getest. Een voor de risicoschatting belangrijk effect is dat de effecten van PCB28 en PCB52 op de $GABA_A$ receptor samen additief zijn in dit *in vitro* systeem. Echter, de experimenten met een binair mengsel bestaande uit PCB28 en PCB153 laten zien dat het versterkende effect van PCB28 wordt verminderd door de hoog gechloreerde PCB153, wat antagonisme of competitieve binding aan de $GABA_A$ receptor kan suggereren. Uit deze experimenten kan dus worden geconcludeerd dat de kwalitatieve en kwantitatieve samenstelling van het NDA-PCB mengsel het uiteindelijke effect op de $GABA_A$ receptor zal bepalen. Bovendien komt uit deze resultaten ook naar voren, dat volledige blootstellingsclassificatie van de NDA-PCBs met weinig chlooratomen in het mengsel, een van de vereiste is voor humane risicobeoordeling van deze groep PCBs.

De plaats van de chlooratomen t.o.v. het effect op de humane $GABA_A$ receptor.

In hoofdstuk twee is met een beperkt aantal congeneren aangetoond dat PCB28 en PCB52 het effect van de postsynaptische $GABA_A$ receptor versterken.
In hoofdstuk drie is een selectie van 20 NDA-PCBs en twee DA-PCBs getest op mogelijke directe effecten op de $GABA_A$ receptor en de daarbij behorende structuur-activiteit-relaties (SAR).

De resultaten laten zien dat de congeneren met weinig chlooratomen PCB19, PCB28, PCB47, PCB51, PCB52, PCB95 en PCB100 bij een lage receptorbezetting als partieel agonist fungeren. Met andere woorden, deze PCBs versterken het effect van de $GABA_A$ receptor wanneer zij samen met de natuurlijke agonist GABA worden toegediend. Daarnaast blijken PCB19, PCB47, PCB51 en PCB100 ook een volledige agonist te zijn, omdat deze congeneren de $GABA_A$ receptor ook activeren in de afwezigheid van GABA.

Deze experimenten tonen dus aan dat een aantal NDA-PCBs het effect van de GABA zelf na kunnen bootsen. Een consequentie hiervan kan zijn dat deze interactie tussen PCBs en de $GABA_A$ receptor ook een gevolg kan hebben voor een indirecte wisselwerking met andere stoffen die aan de $GABA_A$ receptor kunnen binden. Hierbij kan o.a. gedacht worden aan een versterkte werking van andere partiele $GABA_A$ receptor agonisten, zoals neurosteroïden of ethanol.

Uit de resultaten kan tevens afgeleid worden dat activering van $GABA_A$ receptoren concentratie afhankelijk is. De afgeleide SAR toont aan dat het hier in belangrijke mate gaat om NDA-PCBs met 3 tot 5 chlooratomen, die 1 tot 3 *ortho*

posities bezetten. Daarnaast is een gelijk aantal (0 of 1) *meta*-substituties aan beide fenylgroepen van belang met geen aangrenzende *para*- en *meta*-substituties aan dezelfde fenylgroep.

PCB47 is van de onderzochte PCBs de meest potente met een Laagst Waargenomen Effect Concentratie (LWEC) van 10 nM. Het activerende en versterkende effect van PCB47 wordt verminderd wanneer deze samen met PCB19, PCB28, PCB153 of PCB180 wordt blootgesteld aan de $GABA_A$ receptor. Dit duidt op competitieve binding zoals ook door anderen werd gevonden voor interacties met de thyroïdhormoon receptor, oestrogeen receptor en Aryl-Hydrocarbon receptor.

Samenvattend, wijzen de uitkomsten van deze experimenten dus op competitieve binding aan de $GABA_A$ receptor, waarbij de aanwezigheid van minder potente of inactieve NDA-PCBs in het mengsel het effect van de meer potente PCBs kan verminderen.

Verstoring van aromatase activiteit en gen-expressie door NDA-PCBs in de rat

De hypothalamus-hypofyse-bijnier-gonade as (HHBG) as speelt een vitale rol in de huishouding van de geslachtshormonen en is een belangrijke coördinator van het voortplantingsproces op volwassen leeftijd. Ondanks de groeiende aanwijzingen van schadelijke effecten van NDA-PCBs op de hormoonhuishouding, lijken *in vivo* effecten afhankelijk te zijn van de periode van blootstelling en de gebruikte congeneren. De variaties in experimentele opzet, onderzochte eindpunten en zuiverheid van congeneren zorgen er echter voor dat het moeilijk is om eerdere onderzoeken te interpreteren en vergelijken. Daarnaast ontbreekt veelal informatie over het effect van perinatale blootstelling door individuele congeneren op geslachtshormoon producerende enzymen (bijvoorbeeld aromatase) in de verschillende levensfasen.

In hoofdstuk vier wordt het effect van perinatale blootstelling aan twee NDA-PCBs, PCB52 en PCB180, op aromatase in de rat nader beschreven. Beide PCBS komen veel voor in het milieu en de humane voedselketen. Om mogelijke veranderingen in de HHBG as na perinatale blootstelling aan NDA-PCBs te bepalen, werd de activiteit van één van de belangrijkste enzymen in de geslachts-hormoonhuishouding, aromatase of CYP19, in verschillende levensfasen van de rat bestudeerd.

Bij ratten die perinataal waren blootgesteld aan PCB52 resulteerde dit in een toename van aromatase activiteit in de volwassen vrouwelijke bijnieren en eierstokken. Aangezien de aromatase activiteit in de mannelijke bijnieren en testikels niet was beïnvloed door de perinatale blootstelling, wijst dit op een geslachtsafhankelijk effect. Deze effecten waren daarnaast ook leeftijd- en congeneer-afhankelijk en alleen waar te nemen op volwassen leeftijd. Ratten die

waren blootgesteld aan PCB180 lieten geen effect op aromatase activiteit zien. Daarentegen was de genexpressie van de androgeen receptor in het volwassen brein verminderd bij dieren die blootgesteld waren aan PCB180, maar dit werd niet gevonden bij PCB52.

Deze resultaten laten zien dat perinatale blootstelling aan een aantal NDA-PCBs de activiteit van aromatase dus kan veranderen, wat ook nog op volwassen leeftijd is waar te nemen. Hierbij moet echter wel de kanttekening worden gemaakt dat de concentraties waarbij deze veranderingen in aromatase activiteit werden waargenomen vele malen boven de humane achtergrond blootstelling liggen.

Gezien de gevonden verschillen tussen PCB52 en PCB180, is meer onderzoek nodig om te bepalen wat de structuur eisen zijn voor deze PCBs en de interactie met aromatase. Verder, door de *in vivo* biotransformatie van PCBs, zal ook de nodige aandacht moeten worden besteed aan de mogelijke rol van PCB metabolieten.

PCBs en hun metabolieten remmen aromatase en zijn (anti-)glucocorticoïden

De huidige risicobeoordeling van stoffen die mogelijk een verstoring van de hormoonhuishouding kunnen veroorzaken, richt zich voornamelijk op de effecten van de oorspronkelijke (moeder)stof. Echter, biotransformatie in het lichaam zorgt voor een systemische blootstelling aan zowel PCBs als hun metabolieten. In mensen kan prenatale blootstelling aan NDA-PCBs gerelateerd worden aan vertragingen in de pre- of postnatale groei en ontwikkeling van het zenuwstelsel. Het is echter nog steeds niet duidelijk in hoeverre deze effecten worden veroorzaakt door de moederstof of de metabolieten hiervan.

In hoofdstuk vijf wordt beschreven hoe een selectie van twintig NDA-PCBs, vier OH-PCBs en vier milieurelevante $MeSO_2$-PCBs werd gebruikt om de *in vitro* effecten van deze stoffen op de aromatase-activiteit en glucocorticoïd receptor te bepalen. Van alle geteste NDA-PCBs veroorzaakte alleen PCB28 een concentratie afhankelijke remming van de aromatase activiteit in microsomen van de humane placenta. Wanneer er echter een hydroxy groep in het NDA-PCB molecuul aanwezig is dan leidt dit tot een aanzienlijk sterkere remming van de aromatase activiteit. Dit kon met name worden aangetoond met PCB180, waarbij de moederstof geen enkele effect had op de aromatase activiteit, maar de gehydroxyleerde metabolieten juist wel. Deze effecten waren te meten bij LWECs in de lage µM range.

De vier milieurelevante $MeSO_2$-PCBs veroorzaakten eveneens een lichte remming van aromatase bij de hoogst geteste concentratie (12 µM).

Door gebruik te maken van een nieuw ontwikkeld *in* vitro systeem van gist cellen met kunstmatig ingebouwde glucucoricoid receptoren werd aangetoond dat NDA-PCBs zowel een matig agonistisch als een antagonisch effect kunnen veroorzaken. OH-PCBs en $MeSO_2$-PCBs fungeerden als antagonist op de glucocorticoïd receptor, waarbij voor OH-PCBs een LWECs werd vastgesteld in de

lage nM range. De waargenomen anti- glucocorticoïd eigenschappen van $MeSO_2$-PCBs bleken grotendeels veroorzaakt te worden door cytotoxiciteit. Voor zover bekend, zijn de resultaten zoals beschreven in hoofdstuk vijf de eerste, die directe effecten van milieurelevante NDA-PCBs en hun metabolieten beschrijven op de glucocorticoïd receptor. Ondanks dat metabolieten momenteel nog niet worden meegenomen in risicobeoordelingen, kan aan de hand van deze resultaten worden geconcludeerd dat PCB metabolieten een sterkere interactie kunnen hebben op de aromatase activiteit of glucocorticoïd receptor.

PCB's en metabolieten toonden in deze in vitro systemen LWEC's voornamelijk in μM concentraties. Echter, OH-PCB's kunnen de glucocorticoid receptor beinvloeden met nM concentraties.

In humaan serum kunnen OH-PCB's worden gevonden in de lage nM concentraties, wat 10-40% van de totale PCB-concentratie is. Individuele OH-PCB-concentraties zijn kennelijk laag, en verantwoordelijk voor een marge van blootstelling (MVB) tussen de effecten gezien in onze *in vitro* systeem en de blootstelling van de mens.

Echter, ondanks de relatief veilige in de MVB voor deze PCB metabolieten, is het belangrijk om de additieve effecten van PCBs, OH-PCBs en $MeSO_2$-PCBs in het achterhoofd te houden. De gevonden effecten op de aromatase activiteit en glucocorticoïd receptor kunnen dus mogelijk een belangrijke rol spelen in de eerder beschreven verstoringen van de geslachtshormoon huishouding.

Belangrijkste conclusies

In dit proefschrift is een serie experimenten beschreven waarbij de effecten van niet-dioxine-achtige-PCBs en hun metabolieten op het neuro-endocriene systeem zijn onderzocht. De directe effecten van NDA-PCBs op het activeren en versterking van de postsynaptische $GABA_A$ receptor zijn *in vitro* bepaald. Deze experimenten werden ook met binaire mengsels NDA-PCBs uitgevoerd om mogelijke interacties tussen verschillende congeneren te bepalen. Dezelfde selectie NDA-PCBs en metabolieten werd gebruikt om *in vitro* remming van de aromatase activiteit en interactie met de glucocorticoïd receptor te bepalen. Daarnaast zijn ook de *in vivo* effecten van perinatale blootstelling aan PCB52 en PCB180, in de rat op aromatase en de androgeen receptor onderzocht.

De belangrijkste resultaten van dit promotie onderzoek zijn:

- Laag gechloreerde, milieu relevante NDA-PCBs kunnen de $GABA_A$ receptor als (partieel) agonist activeren en/of versterken. De structuur-activiteitsrelatie voor deze nieuw gevonden interactie wijkt af van andere belangrijke eindpunten in neurotransmissie.

- Afhankelijk van de mengselsamenstelling kunnen NDA-PCBs de interactie met met de $GABA_A$ receptor op een additieve manier versterken of juist (deels) remmen. De samenstelling van het mengsel bepaalt dus het netto effect op de $GABA_A$ receptor.

- Perinatale blootstelling aan NDA-PCBs kan een congeneer, seks en leeftijd specifiek effect op androgeen receptor expressie in hersenen hebben.

- Perinatale blootstelling aan NDA-PCBs kan een congeneer, seks en leeftijd specifiek effect op de aromatase activiteit in bijnieren en eierstokken hebben.

- NDA-PCBs en de OH-PCBs en $MeSO_2$-PCBs metabolieten zijn *in vitro* remmers van aromatase en de glucocorticoïd receptor.

- Interacties van OH-PCBs met de glucocorticoïd receptor zijn een nog niet eerder beschreven mechanisme.

- De remming van aromatase *in vitro* door PCB metabolieten is sterker dan door de moederstoffen zelf.

- Het verdient aanbeveling om bij de risicobeoordeling van hormoon verstorende stoffen de mogelijke rol van metabolieten van PCBs te betrekken. Het niet meenemen van deze metabolieten kan mogelijk leiden tot een onderschatting van het uiteindelijk effect van NDA-PCBs.

Curriculum Vitae

Elsa Carmélia Antunes Fernandes was born in Seixal, Portugal, on the 26th of October, 1978. Upon completing her secondary education at Escola João de Barros (Corroios) in 1996 she commenced her degree in Food Science and Technology, at the 'Instituto Superior de Agronomia' (ISA) in Lisbon.

In 2002, Elsa relocated to the Netherlands to pursue an ERASMUS student internship at the Postharvest Technology and Fresh Products Group of AFSG (Agrotechnology & Food Sciences Group), at Wageningen University. Over the period of a year she analysed the genetic and physiological ripening process of tomatoes, under controlled atmospheric conditions.

Subsequently, in 2003, under a frame work of the Marie-Curie fellowship she started working at De Smet Engineering S.A. in Belgium, where she was integrated into a R&D project on the application of membrane technology in the edible oil refining process. The following year saw her return to Wageningen University to obtain her MSc. in Food Technology, with a specialisation in Product functionality. Upon completion of her degree Elsa spent a 6 month period at the Institute of Food Safety (RIKILT) under the supervision of Dr. Ron Hoogenboom, during which she focused on the determination of relevant degradation products of Indol-3-carbinol, as natural agonists of the Aryl hydrocarbon receptor.

The year 2006, saw the commencement of the research enclosed in this dissertation at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University under the close supervision of Dr. Majorie van Duursen, Dr. Remco Westerink and Prof. Dr. Martin van den Berg. Though much attention from the scientific community has previously been shed upon Polychlorinated Biphenyls (PCBs), the new findings from this body of work on the Neuroendocrine effects of non-dioxin-like-PCBs were translated into a Otto Hutzinger prize at the DIOXIN (International Symposium on Halogenated Persistent Organic Pollutants), 2009, for best student presentation. Elsa is currently employed as a Post-Doctoral Employee at the Dairy Science and Technology Group of Wageningen University.

List of Publications

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When living abroad, there are many people who contributed (and continue to do so) to make the place we live feel a little bit more like home, and so, of course I've some very special lines that go out to my "troelstraweg family", even if it's only recently that I've moved here, our bond is strong, and difficult to put into words… thanks for the laughter, the support, the many shared cooking and living experiences that surpass this paragraph: Catita, princesa linda, tá quase, só mais um pouquito e depois vês o alívio que é! E já sabes, o que acontecerá… aqui estaremos para ti; Aldana boludita, jeitosa, a fera… many adjectives to describe your personality and even so, are not enough; Martita the gazpacho queen; Sabina our guru who always has the best stories and theories to tell us; Ishay who shares wise words at exact moment, well, depending on the day hehe; Ben and Julia, my sweethearts aunt Elsa just loves you soooooo much!!! Baci (!!); David, oh-maluco coragem para esta recta final! Mahesh, o.h.d.c., "we're not thriving in this environment… but we're smiling"

Chris and Roelofsen family, during the many years you were a constant support and warm company. We've shared much laughter, and perhaps some less pleasant moments, many coffees on a Sunday afternoon, dinners, Sinter-klaas, birthday parties (…) and I've learned to appreciate the Dutch culture thanks to you. The Netherlands would not have been the same if not for you!

I've been living in Wageningen for a long time, therefore I can not name everyone that was important during this period, since the ERASMUS period, and then returning for a MSc and during this PhD phase… Christinaki, it's been such a long time! I miss you a lot, and wish you all the best in MC!!! Even if you're not present on the 8th of July, Dijkgraaf 8A – I need group therapy, many great moments there ☺, Barbara.. não há coincidencias, certo? Quem diria, Corroios, seixal, Almada e Wageningen! Henrique, Jaime, Joãozinho (menino do Paulo hehe – como o mundo é pequeno) obrigada pelas jantaradas. Klaske, Silvie, Krijn, Merijn nice to have you "around" since Dijkgraaf (2002)! Iliana, Alisah, nice and cozy coffees and dinners. Haarweg-153 Lara, Kamil, Mika it was fun and i really enjoyed our dinners. Jeroen, schouderklopje voor alle de gezelligheid!

A toda a minha familia em Portugal: embora só esteja de volta 3-4 vezes ao ano, para mim é como se o tempo nao passasse e o sentimento é como que vos tenha visto e estado com vocês há tão pouco tempo atras. Estar de volta é sempre facil e acolhedor. Para vocês é o mais dificil de escrever os agradecimentos: como se coloca em papel, mais de 30 anos de convivencia? Seriam necessarias inumeras paginas para descrever os jantares de familia, os Natais, as férias passadas, os aniversários,

enfim... Tio Armindo (Que me dá o melhor Moscatel, famoso até na Holanda), tia Cidalia, Xana, Bruno, Guilherme, Diogo, Tomás (os meus meninos lindos), Jorge, Nuno. Obrigada pelos natais, por serem sempre tão acolhedores, e até (ou importantemente) pelo maravilhoso look que trago sempre para a Holanda. Tia Maria, Tio Antonio, Tininha, Francisco, Rodrigo, obrigada pelos bons tempos passados em CB en Pousafoles. Avózinha linda do meu coracão, que me dá o melhores elogios, um beijinho grande. Tia Alzira, Tio João, Zé, um grande beijinho de saudades. Madrinha e padrinho, são um exemplo de empreendimento para com a vossa comunidade e mesmo assim teem sempre tempo para uma boa conversa.

Aos meus amigos... vou-me repetir, mas para vocês é igualmente dificil escrever algo que iguale ou transmita o carinho, as saudades e o conforto que sinto quando vou a Portugal e sei que vos posso encotrar lá. Alguns já vos conheco desde... os meus (vossos) 5-6 anos... e por isso mesmo não se pode descrever todos os bons tempos passados: Ana Luisa, amiguinha, estes ultimos tempos temo-nos visto um pouco menos... Ana Paula e Carlos, os jantares em vossa casa são sempre uma verdadeira maravilha. Desde os tempos do ISA, Ana Carlos, Gigi, André, Miguel B.A, Miguel A. Solange, Sónia, vocês nem imaginam como é bom e reconfortante as idas ao Bairro e belas jantarada, e as conversas que nunca sabemos onde acabam. Bruno, Carla, Amandio João Paulo, Joana, Nuno, Rita, João Pedro, Claudine, Paulinha, Tânia sem vocês estes últimos anos não teriam sido o mesmo. Mais uma vez, os jantares, as passagem de ano, as idas ao “radar” e tudo aquilo que não cabe num livro... um grande beijinho!

O lugar de honra tem de ser para o meu pai, a minha mãe e a minha mana, Belinha, eu sei que nem sempre foi, ou tem sido facil, estar longe de casa e perder ocasiões importantes na vossa vida. Mas durante estes ultimos anos o vosso apoio foi indispensável. Todos sabemos como o inicio do douturamento foi ainda mais dificil e mesmo assim sempre tiveram uma palavra amiga. Obrigada por tudo!

Elsita