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New insights into the endocrine disrupting effects of brominated flame retardants

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ABSTRACT

The objective of this review is to provide an overview of recent studies demonstrating the endocrine disrupting (ED) effects of brominated flame retardants (BFRs), while highlighting interesting data presented at the recent international BFR workshop in Amsterdam in April, 2007. A review written in 2002 was used as a starting point and about 60 publications published since 2003 were reviewed. New insights into the *in vivo* effects of BFRs on thyroid hormone, estrogen and androgen pathways in both mammalian and non-mammalian models are provided, and novel (*in vitro*) findings on the mechanisms underlying ED effects are highlighted. Special attention is also given to reports on neurotoxicological effects at relatively low doses of BFRs, although an endocrine-related mechanism is disputable. Convincing evidence has been published showing that BFRs and importantly, BFR metabolites, have the potential to disrupt endocrine systems at multiple target sites. While some studies suggest a wide margin of safety between effect concentrations in rodent models and levels encountered in humans and the environment, other studies demonstrate that exposure to low doses relevant for humans and wildlife at critical time points in development can result in profound effects on both endocrine pathways and (neuro)development.

1. Introduction

In 2002, a concise review of studies demonstrating the endocrine disrupting (ED) potency of brominated flame retardants was written (Legler and Brouwer, 2003). At that time, most of the effects reported by BFR exposure in *in vivo* and *in vitro* studies involved reduction in circulating thyroid hormone levels, and neurotoxicological effects. Recently in April 2007, the author was asked to present the highlights of the presentations on BFR toxicity at the 4th International BFR workshop in Amsterdam (<http://www.bfr2007.com/>). Throughout this international conference, it was very apparent that considerable progress has been made since 2002 in understanding BFR-induced (endocrine) toxicity and related mechanisms of action. The objective of the present paper is to provide an overview on new insights into the ED effects of BFRs from recent publications dating from January 2003 to January 2008, while highlighting interesting results given in oral presentations at BFR 2007. Approximately 60 publications were reviewed, demonstrating the explosion in research being performed on this topic worldwide. Whereas in the previous review, most studies concentrated on the (lower brominated) polybrominated diphenyl ethers (PBDEs) (Legler and Brouwer, 2003), recent literature shows an increasing number of studies on other environmentally relevant BFRs, including tetrabromobisphenonal A (TBBPA), 1,2,5,6,9,10-hexabromocyclododecane (HBCD) and higher brominated PBDEs including the deca congener 2,2′,3,3′,4,4′,5,5′,6,6-decabrominated diphenyl ether (BDE 209).

As in the previous review in 2003, the same definition of an ED compound has been maintained, namely "an exogenous agent which interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development or behaviour" (Kavlock et al., 1996). This wide definition includes all substances that can affect endocrine function via interference with hormone (e.g. estrogen, androgen, or thyroid hormone) pathways. Recent *in vivo* and *in vitro* studies illustrating effects on thyroid hormones, estrogens, and androgens are outlined below. Also, novel mechanisms of ED activity are presented, and special attention is given to recent reports of neurotoxicity of BFRs and possible mechanisms underlying effects on critical stages of development.

1.1. Interactions of BFRs with thyroid hormone pathways

1.1.1. In vivo rodent studies

At BFR 2007, Dr. L. van der Ven presented the results of the EU project "FIRE", a large integrated project with the goal of assessing the risk of BFRs for humans and ecosystem health (van der Ven et al., 2007; see also http://www.rivm.nl/fire/). In the FIRE project, comprehensive *in vivo* studies were carried out according to (enhanced) OECD protocols with male and female Wistar rats exposed for 28 days to HBCD, TBBPA, BDE 209 and the pentabrominated diphenyl ether mixture (DE-71), which had been subjected to a

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purification step prior to exposure to remove potential dioxin-like contaminants. The thyroid hormone system appeared to be the main target for these compounds; reduction of circulating thyroxin (TT4) was observed with TBBPA and DE-71, while increased levels of circulating triiodothyronine (TT3) were observed with TBBPA and BDE 209 exposure (Van der Ven et al., 2007). In a recent publication on the effects of HBCD 28-day repeated exposure in rats, van der Ven et al. (2006) showed that HBCD-induced dose-related effects on the thyroid hormone axis, including reduction of TT4 at a benchmark dose level (BMDL) of 55.5 mg/kg/day, increased pituitary weight at a BMDL of 29 mg/kg/day, increased immunostaining of TSH in the pituitary, increased thyroid weight (at a very low BMDL of 1.6 mg/kg/day), and thyroid follicle cell activation. Interestingly, these effects were found in female rats only. As recent exposure data in human breast milk show HBCD levels that are at least four orders of magnitude below the lowest BMDL shown in this study, the authors conclude that their study does not support a major health risk of HBCD for humans (van der Ven et al., 2006).

The effects on thyroid hormones shown by van der Ven and coworkers following exposure of rats to DE-71 corroborate results presented in earlier studies (Stoker et al., 2004; Ellis-Hutchings et al., 2006). In the Stoker et al. study, in which lower concentrations of DE-71 (3, 30 and 60 mg/kg/day) were used, serum T4 was significantly decreased in male and female Wistar rats following 5 day exposure in males and females, and in 21-day exposed females. Doses of 3, 30, and 60 mg/kg/day DE-71 decreased T4 in 31-day exposed males. Serum T3 was decreased and TSH elevated by 30 and 60 mg/kg in the 31-day exposed males only. Also, a liver biotransformation enzyme involved in T4 metabolism, uridinediphosphate-glucuronosyltransferase (UDGPT), as well as ethoxy- and pentoxy-resorufin-O-deethylase (EROD and PROD), were induced at the two highest doses in all exposures (Stoker et al., 2004). The induction of EROD may indicate a contamination of the commercial DE-71 mixture with polybrominated dibenzofurans as reported by Sanders et al. (2005). In a study designed to assess the effects of maternal gestational and lactational DE-71 exposure on thyroid hormone and vitamin A homeostasis, rats of sufficient vitamin A (VAS) or marginal vitamin A (VAM) status and their offspring were exposed orally to 18 mg/kg/bw/day DE-71 from gestation day (GD) 6 to lactation day 18 (Ellis-Hutchings et al., 2006). DE-71 exposure lowered plasma T4 concentrations in both VAS and VAM dams and pups, while plasma thyroid stimulating hormone (TSH) concentrations were high in exposed VAM dams exposed to DE-71. These novel results suggest that marginal vitamin A status, which may often occur in pregnant women, enhances the susceptibility to thyroid hormone axis disruption by DE-71 (Ellis-Hutchings et al., 2006).

Additional studies have also revealed the thyroid hormone disrupting effects of other BFRs in rodents. Kuriyama et al. (2007) have recently reported that low doses of 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) reduce T4 in Wistar rats. The doses selected were close to those reported for BDE-99 in non-occupationally exposed humans. A single dose of 300 μg BDE-99/kg b.w. at GD 6 reduced T4 concentrations in dams at the beginning of lactation (post gestational day (PGD) 1), and slightly reduced T4 at PGD 22. In offspring, reduced T4 was observed at postnatal day (PND) 22. Importantly, these authors have previously reported that this BDE-99 exposure concentration, and even a lower (60 μg/kg bw) exposure concentration, during rat development causes neurotoxicological effects (i.e. hyperactivity) and reproductive effects (i.e. impaired spermatogenesis) (Kuriyama et al., 2005). In a very recent study, Richardson et al. (2008) found significantly decreased plasma T4 concentrations in female C57BL/6 mice exposed to 100 mg/kg/day of BDE-47 for 4 days. Results from this study indicate novel mechanisms of decreased circulating T4: in addition to induction of hepatic Ugt1a1, Ugt1a7, and Ugt2b5 mRNA expression which implies increased elimination of T4, BDE-47 exposure decreased the mRNA expression of hepatic transthyretin (TTR) and a specific TH transporter monocarboxylate transporter 8 (Mct8) mRNA (Richardson et al., 2008). Mct8 is important for membrane transport and cellular entry of TH, which is required for conversion of thyroid hormone by intracellular deiodinases and for binding T3 to its nuclear receptors (Jansen et al., 2005).

1.1.2. In vivo studies with aquatic organisms

In another presentation of results from the FIRE project at BFR 2007, Dr. R. Kuiper showed that exposure of DE-71 in two fish models, the European flounder (*Platichthys flesus)* and the zebrafish (*Danio rerio*), resulted in modest effects on thyroid hormones (Kuiper et al., 2007a). In flounder exposed for 101 days to food and sediment spiked with DE-71, a significant (10%) reduction in plasma T4 was found at an internal dose of 51 ng BDE-47/g wet weight (w.w.). In contrast, in zebrafish adults and juveniles exposed in a partial life cycle study, a mild increase of plasma thyroid hormone levels was found with increasing internal PBDE concentrations. The internal levels in the zebrafish were much higher than those reached in the flounder, which may explain the differences in effects on T4 (Kuiper et al., 2007a). Kuiper and colleagues have also recently demonstrated that exposure of juvenile flounder to waterborne TBBPA for 105 days results in increased plasma T4, but not T3, levels at internal concentrations of about 90 ng/g w.w. or higher (Kuiper et al., 2007b). HBCD exposure with resulting internal concentrations of up to 446 μg/g lipid weight, however, did not result in effects on thyroid hormones in the flounder (Kuiper et al., 2007b), indicating that the marine species may be less sensitive than the tropical freshwater species. In a presentation by Dr. V. Palace at BFR, 2007, prolonged (56 day) exposure of juvenile rainbow trout to the three diastereoisomers of HBCD (α, β, γ-HBCD) did result in indications of thyroidal effects; enlarged thyroid epithelial cells (a measure of thyroid gland hypertrophy) were found following exposure to γ-HBCD (Palace et al., 2007). In a comparable study by the same authors, exposure of juvenile rainbow trout to a mixture of 13 environmentally relevant PBDEs resulted in decreased T4 plasma levels after 56 days of exposure to 2.5 and 25 ng/g food (Tomy et al., 2004).

A number of *in vivo* studies in amphibians have been recently published which show that BFRs can interfere with thyroid hormone-related processes in development. At BFR 2007, Dr. A.J. Murk presented work from her group with a novel *Xenopus laevis* tadpole tale tip bioassay and complementary *in vitro* bioassays (Murk et al., 2007). Tadpole tail tips cultured *ex vivo* respond to T3 by undergoing TR-mediated regression, mimicking the TH-regulated resorption of primary tissues during metamorphosis. Exposure of tadpole tail tips to BDE206 at concentrations of 10 nM or higher antagonized T3-mediated tail resorption, while HBCD (1 μM), when exposed together with the EC50 value of T3, enhanced the effects of T3 (Schriks et al., 2006a). Balch et al. (2006) have also shown that exposure of *Xenopus laevis* tadpoles to BDE-47 or DE-71 through the diet or directly by injection results in inhibition of tail resorption, delayed metamorphosis and impacts on skin pigmentation, indicating disruption of TH homeostasis during development. In another novel *Xenopus laevis* bioassay, Fini et al. (2007) developed transgenic embryos carrying a fluorescent TH/bZIP-eGFP construct, which contains a consensus thyroid hormone response element (TRE). The transgenic tadpole bioassay responds in particular to activation of the thyroid hormone receptor TRβ. Screening of transgenic embryos in 96-well plates revealed that TBBPA is a TRβ antagonist, with a 1 μM exposure inhibiting T3-mediated fluorescence by 20% (Fini et al., 2007). Caution should be exercised when interpreting these effects as ED at these high concentrations, however, as a recent study in human HepG2 cells

showed effects of TBBPA on cell membrane integrity (Ogunbayo et al., 2007). A recent study with the Pacific tree frog, *Pseudacris regilla*, showed that TBBPA exposure at low concentrations (10–100 nM) in larval stages actually enhanced TH-mediated effects (Veldhoen et al., 2006). Tadpoles exposed to 10 nM TBBPA for 48 h showed an increase in gelatinase B mRNA in the tadpole tail which was associated with increased tail resorption by 96 h. TH-mediated TRα mRNA expression in the tadpole brain was increased by 100 nM TBBPA exposure, while TBBPA alone altered TRα mRNA expression in the tail. The expression of TRβ mRNA was not affected by exposure to TBBPA either alone or in the presence of TH (Veldhoen et al., 2006).

1.1.3. In vivo studies with birds

A series of unique studies have been carried out to determine the effects of BDEs on American kestrels (*Falco sparverius*) (Fernie 2005, 2007, 2008). In the Fernie et al. (2005) study, kestrel eggs were injected with a mixture of BDE-47, 99, 100, and 153 (concentration of total BDEs 1000 ng/g) and nestlings from dosed eggs were orally exposed for 29 days to the mixture at levels approximating those reported in Great Lakes trout (100 ng/g). Kestrels exposed to the BDE mixture showed lower plasma T4 levels, plasma retinol, and hepatic retinol and retinyl palmitate concentrations, but unaltered T3 concentrations and thyroid glandular structure (Fernie et al., 2005). In consequent studies recently published and presented at the BFR 2007 conference, adult kestrels were exposed to DE-71 for 75 days through the diet (0.3 or 1.6 ppm) (Fernie et al., 2007, 2008). Dramatic effects on reproductive behaviour were found in exposed kestrels, with altered courtship behaviour and compromised strength of the pair-bond (Fernie et al., 2008). Consequent effects on reproductive fitness were found in kestrels exposed to low- or high-doses of DE-71: delayed clutches, smaller eggs, and reductions in fertility, hatching success, and fledging success compared to the control pairs (Fernie et al., 2007). These important results show that exposure to BDEs at environmental levels not only affects thyroid hormone homeostasis in birds, but can also lead to altered reproductive behaviour and fitness, and adverse effects on the growth of nestlings.

1.1.4. In vitro mechanistic studies

In vitro studies performed in recent years have provided a wealth of information to support the thyroid hormone-mediated effects of BFRs observed *in vivo*, as well as to reveal new potential mechanisms of action. At BFR 2007, Dr. T. Hamers provided compelling evidence that the HBCD-induced decrease in T4 levels seen *in vivo* (van der Ven et al., 2006) may be (partly) caused by an increase in tissue T4 uptake by membrane proteins involved in TH transport. *In vitro* studies indicate that HBCD increases mRNA expression of one member of the organic anion transporting polypeptides (OATP) family of homologous membrane bound solute carrier proteins in a rat liver cell line (Kamstra et al., 2007). Future studies should reveal the importance of OATP induction in the *in vivo* TH-mediated effects of HBCD. *In vitro* studies with the GH3 rat pituitary cell line ("T-screen") also confirm effects seen the *in vivo* tadpole tail tip assay (Schriks et al., 2006a): BDE206 antagonizes T3-mediated cell proliferation, while HBCD co-administration enhances the effect of T3 on cell proliferation (Schriks et al., 2006b). HBCD "potentiation" of T3-mediated effects has also been observed in HeLaTR cells transfected with a TRE-luciferase construct (Yamada-Okabe et al., 2005). In green monkey kidney fibroblast (CV-1) cells transiently transfected with *Xenopus* TRα or TRβ and a luciferase reporter gene, however, HBCD was inactive, suggesting that HBCD potentiation is not regulated at the level of the (*Xenopus*) TR (Schriks et al., 2007). This study also revealed TRα-specific (diiodobiphenyl) and TRβ-specific (BDE 28) ligands (Schriks et al., 2007). Similar to effects seen *in vivo* in tree frogs, *in vitro* studies with the T-screen have shown TBBPA to be a TH agonist, inducing cell proliferation of GH3 cells (Kitamura et al., 2005). In a recent study, Kudo et al. (2007) have shown in *in vitro* binding and transactivation studies that a brominated derivative of bisphenol A, 3,3′,5-tribromobisphenol A, potently binds the *Xenopus* TH plasma transport protein transthyretin (TTR) and activates *Xenopus* TR, while it induces TRβ and TH/bZIP gene (mRNA) expression in *Xenopus laevis* embryos *in vivo*.

In a comprehensive study of the *in vitro* endocrine disrupting potency of various BFRs, Hamers et al. (2006) recently confirmed the TH-disrupting activity (binding to TTR) of TBBPA, 2,4,6-tribromophenol and the hydroxylated BDE-47 metabolite 6-OH-BDE-47 while revealing a number of novel mechanisms of endocrine disruption (see below). In subsequent research aimed at examining the TTR-binding activity of BFR metabolites formed by microsomal incubation, Hamers et al. (2008) have shown that many PBDE metabolites have higher TTR-binding potencies than the parent compounds. Microsomal incubation of BDE-47 led to the identification of six hydroxylated metabolites, which showed TTR-binding potencies 160–1600 higher than BDE-47 itself. This study, and further studies with metabolites shown below, highlights the importance of considering metabolites in the assessment of BFR risk. Accordingly, a kinetic study with pregnant Wistar rats exposed to BDE 209 has shown that several metabolites are present both in maternal tissues and in foetuses, including three nona-BDEs and one octaBDE (Riu et al., 2008). Obviously, BFR metabolites can pass the placenta where they may pose a risk for the developing embryo. It should be noted, however, that some of the hydroxylated BDEs may also be naturally produced from aquatic organisms such as algae (Malmvärn et al., 2005). The predominant structure of the naturally occurring compounds has the OH-group attached to an *ortho*-position. For the PBDE metabolites, the OH-group is mainly in the *meta*- and *para*-positions (Malmberg et al., 2005).

1.2. Interactions of BFRs with estrogen pathways

1.2.1. In vivo rodent studies

Three studies reporting the estrogenic effects of BDE-99 exposure in rats have been recently published. Talsness et al (2005) exposed pregnant Wistar rats by gavage to a single dose of 60 or 300 μg/kg bw at GD 6, which resulted in female reproductive tract changes in the F1 generation which were apparent at adulthood, i.e. ultrastructural changes in the ovaries. In a study in which adult female Long-Evans rats were exposed by subcutaneous injection to 1 or 10 mg/kg/day from GD10-18, offspring exhibited unchanged uterine weight but increased ovarian weight (Ceccatelli et al., 2006). Importantly, the authors showed that prenatal exposure to BDE-99 can disrupt the expression of estrogen target genes and their regulation by endogenous estrogens. To this end, down-regulation of progesterone receptor (PR) mRNA was found in offspring at both BDE-99 doses, while estrogen receptor alpha (ERα), ERβ and insulin-like growth factor-I (IGF-I) were upregulated at the lower dose (Ceccatelli et al., 2006). In a third study with Long-Evans hooded rats exposed in a similar fashion to the same concentrations, some effects were more profound in male offspring, e.g. decreases in circulating sex steroids (estradiol (E2) and testosterone (T)) at weaning and in adulthood, reduction of anogenital distance and feminization of sexually dimorphic behaviour (Lillienthal et al., 2006). In female offspring, puberty onset was delayed at the higher dose level, whereas a slight acceleration was detected in low-dose males. The number of primordial/primary ovarian follicles was reduced in females at the lower dose, whereas decline of secondary follicles was more pronounced at the higher dose (Lillienthal et al., 2006).

In addition to the BDE-99 studies, a recent study of the effects of BDE-47 exposure in rats has shown the induction of a novel

biomarker of estrogen action, namely calbindin-D9k (CaBP-9k), a vitamin D–dependent calcium-binding protein whose expression in rat uteri is upregulated by estrogen and downregulated by progesterone during the estrous cycle and early pregnancy (Dang et al., 2007). Exposure (200 mg/kg/day, injection) of juvenile rats resulted in a signification increase in uterine CaBP-9k mRNA and protein 24 h after injection, as well as a clear uterotrophic response, which was reversed by anti-estrogen treatment (Dang et al., 2007). These results indicate that BDE-47 may elicit effects on development of the female reproductive system. This effect is particularly relevant in the light of recent toxicokinetic studies, which have revealed that in C57BL/6 mice exposed to a single oral dose of 1 mg/kg BDE-47, the toxicokinetics of BDE-47 are different in developing mice than in adult mice (Staskal et al., 2006). Developing mice have a reduced capacity to excrete BDE-47, which leads to higher concentrations in target tissues during critical windows of development (Staskal et al., 2006).

1.2.2. In vivo studies with aquatic organisms

Recent studies in fish exposed to HBCD and TBBPA indicate that these BFRs do not elicit estrogenic effects. Juvenile rainbow trout (*Oncorhynchus mykiss*) and feral eelpout (*Zoarces viviparus*) exposed via interperitoineal injection of HBCD and TBBPA (up to 100 and 500 mg/kg respectively) did not show any effects on vitellogenin plasma protein levels (Ronisz et al., 2004). Similarly, long term exposure of European flounder and zebrafish to these compounds did not induce vitellogenic responses (Kuiper et al., 2007a). An additional study has also revealed that exposure of estrogen-responsive transgenic "reporter" zebrafish to TBBPA, HBCD, BDE-47 as well as BDE 209 through the water or food phase does not result in expression of an estrogen receptor-mediated luciferase reporter construct (Legler et al., 2005).

1.2.3. In vitro mechanistic studies

Lack of estrogenic effects of HBCD and TBBPA have also been demonstrated in *in vitro* reporter gene assays in human T47D cells (ER-CALUX; Hamers et al., 2006) and cell proliferation assays in human MCF-7 cells (Olsen et al., 2003). Exposure of yeast reporter cells containing a human estrogen receptor gene did not result in reporter gene activity by BDE-47, BDE-99, BDE-205, PBB-153 and the technical Firemaster BP-6TM (Nakari and Pessala, 2005). These BDEs also do not induce reporter gene activity in the ER-CALUX assay (Hamers et al., 2006). In contrast, exposure of freshly isolated fish hepatocytes to these chemicals and measurement of vitellogenin showed clear estrogenic effects of all BDEs tested (Nakari and Pessala, 2005). "U-shaped" dose–response curves shown were attributed to cytotoxicity at higher concentrations. The discrepancy between estrogenic responses in yeast and human cell lines as compared to the fish hepatocytes may be attributed to the high metabolic capacity of the fish cells and consequent production of estrogenic metabolites. Hamers et al. (2008) recently demonstrated that BDE metabolites produced by microsomal incubation of parent compounds have 2–200 times higher potency to inhibit estradiol sulfotransferase, an enzyme responsible for the sulfonation and subsequent inactivation of the endogenous hormone estradiol.

In vitro anti-estrogenic effects have been reported for the hydroxylated BDE-47 metabolite 6-OH-BDE-47 (Hamers et al., 2006). This metabolite also appeared to inhibit the estradiol-mediated induction of a luciferase reporter gene in a transgenic zebrafish model (Legler et al., 2005). However, this metabolite is acutely toxic in fish through uncoupling of oxidative phosphorylation (van Boxtel et al., 2008) and shows cytotoxicity *in vitro* as well (Hamers et al., 2006), making it difficult to demonstrate its anti-estrogenic properties conclusively.

Interestingly, in one of the few studies to examine estrogenic effects of BFRs in invertebrates, Wollenberger et al. (2005), have shown that some BDEs can inhibit larval development of the marine copepod *Acartia tonsa* at low mg/l concentrations. *In vitro* assessment of the BFRs for ecdysteroid agonistic/antagonistic activity was carried out with the ecdysteroid-responsive Drosophila melanogaster BII-cell line, and revealed that BDE-99 and BDE 100 have weak ecdysteroid antagonistic activity (Wollenberger et al., 2005).

1.3. Interactions of BFRs with androgen pathways

1.3.1. In vivo rodent studies

A number of studies have been recently published that indicate that BFRs may disrupt androgen homeostasis and male reproductive organ development. At the BFR 2007, results of the FIRE *in vivo* studies presented by Dr. L. van der Ven indicated not only effects on the thyroid system, but also on the androgen system (Van der Ven et al., 2007). TBBPA exposure in rats resulted in an increase of gonad weights in F1 males, and effects on circulating testosterone in males, aromatase activity in the ovary, and pituitary weights in males. BDE 209 and DE-71 exposure caused a decrease in male accessory reproductive organs and induction of androgen synthesis in female adrenals (Van der Ven et al., 2007). These apparent anti-androgenic effects of DE-71 corroborate a previous study by Stoker et al. (2005), in which juvenile Wister rats exposed to 60 and 120 mg/kg/day of DE-71 for 31 days showed a delay in preputial separation (PPS) (a marker of puberty onset) and reduced ventral prostate (VP) and seminal vesicle growth at both doses. Adult males exposed to 60 mg/kg DE-71 for 3 days showed a significant increase in luteinizing hormone and a non-significant increase in testosterone, androstenedione and estrone. DE-71 also tested positive for anti-androgenic activity in an immature rat Hershberger assay, with doses of 30–240 mg/kg resulting in decreased VP and seminal vesicle weight (Stoker et al., 2005). Tseng et al. (2006), however, have demonstrated that exposure of mice to BDE 209 at high concentrations of 500- and 1500-mg/kg/day from PND 21-70 did not affect sperm count or function, though indications of oxidative stress in sperm were found.

1.3.2. In vitro mechanistic studies

The anti-androgenic effects of DE-71 seen *in vivo* have been confirmed with various *in vitro* studies. DE-71 and BDE 100 have been found to inhibit binding to the androgen receptor (AR) (Stoker et al., 2005). In addition to DE-71, BDE 100 and BDE-47 inhibit the dihydrotestosterone (DHT)-mediated transcription in the MDA-kb2 cell line containing an endogenous human AR and a luciferase reporter gene (Stoker et al., 2005). In a large *in vitro* screen, AR-antagonistic potencies have been found for 18 out of 27 BFRs (Hamers et al., 2006). In fact, both BDE 19 and BDE 100 have a higher anti-androgenic activity than the prostate therapeutic flutamide (IC50 60 and 100 nM, respectively) (Hamers et al., 2006). Quantitative structure–activity relationship models based on androgen antagonism have been carried out, and suggest that lower brominated PBDEs with bromine substitutions in *ortho*-positions and bromine-free *meta*- and *para*-positions have the highest potencies (Harju et al., 2007).

Interestingly, in a modelling study of various BFRs, new and convincing evidence has been provided showing that 1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane (BCH), a BFR used in construction material, plastic parts of appliances, and electric cable coating, is an androgen *agonist* (Larsson et al., 2006). BCH binds to the AR and induces luciferase in human hepatocellular liver carcinoma cells (HepG2), transiently transfected with the human AR and a luciferase reporter gene at low μM concentrations. Furthermore, modelling studies of the AR showed that BCD preferentially be located in the ligand pocket (Larsson et al., 2006). Further *in vivo*

studies with BCH are required to assess if BCH is androgenic at environmentally relevant concentrations.

In addition to direct effects through (ant)agonism of androgen receptors, recent studies *in vitro* have also indicated an alternative mechanism to explain the (anti-)androgenic effects observed *in vivo*. Some BFRs and their metabolites may induce or inhibit steroidogenic enzymes important in the conversion of testosterone to estradiol (aromatase, CYP19) and in the biosynthesis of the weak androgens dehydroepiandrosterone (DHEA) and androstenedione in the adrenals, as well as testosterone in the testis (CYP 17). CYP17 also catalyzes the conversion of aldosterone to corticosteroid substrates, important for cortisol synthesis in the adrenal glands. Studies in the H295R human adrenocortical carcinoma cells have shown that some hydroxylated and methoxlyated BDE metabolites (6-OH-BDE-47, 6-OH-BDE-99, 6-MeO-BDE-47) inhibit CYP 19 at low μM concentrations, while 2,4,6-tribromophenol induces this enzyme (Cantón et al., 2005). In human placental microsomes, this group of researchers has also shown that 11 hydroxylated BDEs all inhibit of placental aromatase activity with IC50 values in the low micromolar range (Cantón et al., 2008). The position of the OH-group, either ortho or meta, appears to have no effect on the inhibition of aromatase. In addition to inhibiting aromatase, studies in the H295R cell line have also demonstrated that hydroxylated BDEs inhibit CYP 17, though cytotoxic effects at high concentrations may confound this effect (Cantón et al., 2006). In another study in H295R cells, Ding et al. (2007) have also shown that 2,4,6-TBP suppresses CYP17 mRNA expression. This study also revealed that the most highly upregulated gene in the H295R cells exposed to various bromophenols including 2,4,6-TBP was the 3β-hydroxysteroid dehydrogenase isomerase gene (*3β-HSD2*). The gene is responsible for conversion of 5-ene-3β-hydroxy steroids to 4-ene-3-ketosteroids, an obligate step in the biosynthesis of both androgens and estrogens, as well as mineralocorticoids and glucocorticoids (Ding et al., 2007).

1.4. Steroid hormone metabolism

Recent studies have been pivotal in demonstrating a novel mechanism of action of some BFRs to explain the induction of CYP3A and CYP2B observed *in vivo*: induction of the pregnane X receptor (PXR, called steroid X receptor (SXR) in humans) and the constitutive androstane receptor (CAR). Induction of these receptors, and their related CYPs, may affect the homeostasis of endogenous substances including steroid and thyroid hormones. Pacyniak et al. (2007) have provided convincing evidence of the role of PXR in BFR-related effects, as treatment of C57BL6 mice with BDE-47, 99, and 209 induced gene expression of cyp3a11 and 2b10, but not cyp1a1/2. In PXR knockout mice, induction of cyp3a11 and 2b10 by these BDEs was suppressed. *In vitro* transactivation assays confirmed that BDE-47, 99, and 209 activated PXR and SXR, but not aryl hydrocarbon receptor (Pacyniak et al., 2007). Germer et al. (2006) have also shown that exposure to HBCD, but not TBBPA, induces CYP2B1 mRNA, CYP2B1/2B2 protein and 7-pentoxyresorufin O-dependentylase (PROD) activity in rats exposed for 28 days, as well as inducing CYP3A1/3A3 mRNA, CYP3A1 protein, and luciferin benzylether debenzylase (LBD) activity. At BFR 2007, induction of Cyp2B and Cyp3A mRNA expression in hepatocytes exposed to a technical BDE-47 mixture was also presented by M. Wahl (Wahl et al., 2007). Induction of Cyp1A in the zebrafish embryos was attributed to contamination of the technical mixture with dioxin-like brominated furans. Similarly, Sanders et al. (2005) showed that BDE-47, 99 and 153 upregulated CYP2B and CYP3A gene expression in rat livers at doses similar to that for non-dioxin-like PCB153. These studies suggest the involvement of the CAR/PXR signalling pathway in the induction of these steroid-metabolizing enzymes.

1.5. Effects of BFRs on (brain) development

Although it can be disputed if the effects of BFRs on neurological development are hormone-mediated and therefore endocrine disrupting according to the definition given in the introduction, studies on these effects deserve special attention here given this critical stage of development. Significant progress has been made in demonstrating the effects, and underlying mechanisms, of BFRs in the developing rodent brain, at levels that tend to be lower than reported for the endocrine disrupting effects shown above. In studies with BDE 209, Viberg et al. (2003) found changes in spontaneous behaviour in mice treated on GD 3 with 2 and 20 mg/kg/bw, effects that worsened with age. In a follow-up study in rats given a similar dose, similar neurobehavioural effects were found, with evidence that this BDE, and others lower brominated BDEs, disturb the cholinergic system (Viberg et al., 2007). Studies with radiolabelled BDE 209 showed the presence of this compound in the mouse neonatal brain (Viberg et al., 2003). To determine the effects of debrominated metabolites of BDE 209, Viberg et al. (2006) exposed mice at GD 3 to nonaBDE (BDE 206), octaBDE (BDE 203), and heptaBDE (BDE 183), and found that these three decaBDE metabolites can induce developmental neurotoxic effects. In particular BDE 203, when administered on GD 3 or 10 at a concentration of 21 mmol/kg bw, caused effects at 2 months of age, such as disturbances in spontaneous behaviour, disrupted habituation and hyperactivity. Exposure to BDE 203 at GD 10 also affected learning and memory functions in adult mice (Viberg et al., 2006). Hyperactivity has also been demonstrated in mice exposed to a single dose of BDE-47 (1, 10, or 30 mg/kg) during postnatal development (Gee and Moser, 2008). Eriksson et al. (2006) have demonstrated earlier that exposure to mice at PND 10–6.8 mg/kg/bw caused effects on learning and memory. At the same dose, Dingemans et al. (2007) have provided convincing evidence for the mechanism underlying these effects: changes in the levels of postsynaptic proteins involved in synaptic plasticity in the mouse hippocampus. Neurodevelopmental effects have also been reported for BDE-99 (Branchi et al, 2005; Eriksson et al., 2006; Viberg et al., 2006). An interesting proteomics study in mice exposed to a single dose of 12 mg BDE-99/kg/bw at PND 10 revealed differentially expressed proteins in two regions of the brain (striatus and hippocampus), indicating that BDE-99 may induce cellular stress, neurodegeneration and aberrant neuroplasticity, which may explain neurobehavioural effects observed in adult mice (Alm et al., 2006). New evidence has also been provided for the effects of BFRs on neurotransmitters in the rodent brain: HBCD has been found to inhibit plasma membrane uptake of dopamine uptake in rat brain synaptosomes cultured *ex vivo* (Mariussen and Fonnun, 2003). TBBPA inhibits dopamine, glutamate and γ-amino-*n*-butyric acid (GABA) uptake. Both compounds demonstrate effects on neurotransmitter uptake at a concentration level similar to what previously found for polychlorinated biphenyls (low μM, Mariussen and Fonnun, 2003).

1.6. Concluding remarks

The plethora of recent publications on the effects of BFRs provides convincing evidence that BFRs have the potential to disrupt endocrine systems at multiple target sites, at least in the laboratory situation. While the evidence for thyroid hormone disrupting potency of BFRs has increased, new data published since the Legler and Brouwer (2003) review has clearly demonstrated *in vivo* effects on both the estrogen- and androgen-mediated processes as well. In particular, the anti-androgenic effects of some BFRs shown in both *in vitro* and *in vivo* studies are noteworthy. New (*in vitro*) mechanistic data has indicated novel targets for BFR action, including thyroid hormone-mediated amphibian metamorphosis, enhanced tissue uptake of thyroid hormones, and altered

steroidogenesis and metabolism. While some studies suggest a large margin of safety between effects concentrations in rodent models and measured levels in humans and wildlife, other studies demonstrate that exposure to low doses relevant for humans at critical time points in development can result in profound effects on both the endocrine system and neurodevelopment. Importantly, as shown above, exposure of American kestrels in the laboratory to environmentally relevant concentrations of BDEs has shown dramatic effects on thyroid hormones, reproductive behaviour and outcome (Fernie et al., 2005, 2007, 2008). It is now imperative to demonstrate a relationship between chronic exposure of wildlife to BFRs in the field and endocrine disrupting effects The importance of studying the effects of metabolites of BFRs cannot be stressed enough, as they demonstrate various ED properties, at least *in vitro*: binding to TTR, inhibition of enzymes involved in the metabolism of steroid hormones (e.g. estradiol sulfotransferation), and inhibition of steroidogenesis (e.g. aromatase, CYP17, and 3BHSD). Convincing evidence of ED properties of the lesser-studied BFRs such as HBCD warrants research in the endocrine effects of alternative BFRs. Two examples are BCH, which showed androgenic activity *in vitro* (Larsson et al., 2006), and TBBPA-bis[2,3-dibromopropyl ether],2,2-bis[3,5-dibromo-4-(2,3-dibromopropoxy)phenyl]propane (TBBPA-DBPE), which is slowly metabolized *in vivo* (Knudsen et al., 2007) and shows *in vitro* TTR-binding and E2-sult inhibiting potency (Hamers et al., 2006).

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