CHAPTER 4: DISCUSSION

Increasing exposure to several man-made chemicals after the industrial revolution required the establishment of risk assessment procedures to establish safety levels for human exposure. By definition, risk assessment is the systematic scientific characterization of potential adverse health effects resulting from human exposures to hazardous agents or situations [NRC, 1983]. The most convincing evidence for human risk is a well-conducted epidemiological study in which a positive association between exposure and disease has been observed [NRC, 1983]. However, human exposure data for the prediction of human response

are usually limited and liable to uncertainties. Variables like: multiple exposure (combined effects), small number of individuals investigated, genetic variability and lifestyle factors account for uncertainties [Faustman and Omenn, 1995]. Thus, animal bioassay data are key component of the hazard identification process.

Determination of acceptable human exposure levels for non-carcinogenic effects normally involves extrapolation factors to bridge knowledge gaps from animal studies (*e.g.* dose response relationship, intra- and interspecies difference, population sensitivity and different pathways of uptake) [Kalberlah *et al.*, 2003]. This most evident manifestation of the toxicological concept of adverse implies that toxicology is an above NOAEL discipline because it is the study of "adverse effects." It also implies that either there are no effects below the NOAEL or that they are not relevant to a toxicological analysis. However, a strategy in toxicological evaluations of the last decades has been to compensate for small numbers of experimental animals by using excessive doses. This approach is pragmatic and easy to perform, but there is increasing concern that this might be wrong and unsuitable for risk assessment, given rise to misinterpretation. Such strategy is used to a lesser extent for therapeutic substances, because here the dose range used in experimental studies often does not deviate that much from the anticipated human exposure, especially when serum concentrations are compared rather than doses. However, evaluating environmental chemicals doses in testing and human exposure often diverge by three or more orders of magnitude [Neubert *et al.*, 1992]. For most non-carcinogenic compounds, it is assumed that their toxicity is expressed beyond a certain threshold and the toxic effect increases with increasing dose until a certain maximum dose level. Below the threshold dose, no chronic toxic effect occurs, implying that this dose poses no toxicity for human population [Faustman and Omenn, 1995]. For assessing a safety limit with regard to human health, this threshold dose is divided by a factor of 10 in order to account for interspecies differences in susceptibility (assuming that humans are more sensitive than laboratory animals), and another factor of 10 is used to account for interindividual differences. This yields in point estimates for human limit values like, acceptable daily intakes (ADI) or reference doses (RfD) [Faustman and Omenn, 1995]. The information provided by these point estimates is simple: exposures above the human limit value may be regarded as "unsafe", exposures below as "safe". However, ADI and RfD are always estimated based on high dose studies from animal assays, which are conducted far above the low environmental levels characteristic for human exposure scenarios [Calabrese, 2004; Calabrese, 2003]. Therefore, low-dose extrapolation

and animal-to-human risk extrapolation methods are required for regulatory purposes and constitute major aspects of dose-response assessment [Faustman and Omenn, 1995]. In the last years, experimental observation of adverse effects in the low dose range - typically below the threshold dose or close to human exposure – brought new discussions on risk assessment paradigms.

The effects of low dose exposures to environmental pollutants are a matter of particular importance for regulatory agencies. A low dose effect is defined as biological or physiological changes that occur in the range of human exposures or at doses lower than those typically used in the standard testing paradigm of the regulatory agencies [Melnick *et al.,* 2002]. A peer review panel on endocrine-disruptor chemicals concluded that low dose effects have been demonstrated in laboratory animals and recommended that the current test paradigm for reproductive and developmental assessment of EACs should be revisited [Melnick *et al*., 2002]. Moreover, the fundamental nature of dose response relationships (either linear or threshold) assumed by regulatory agencies have been challenged by experimental demonstration of non-linearity at dose ranges below the NOAEL. In the context of human risk assessment, the present study represents an important observation as offspring rat exposed to PBDE 99 doses resembling human exposure were adversely affected in different endpoints assessed. In this chapter, important aspects of toxicity induced by PBDE 99 in an animal experiment model are discussed.

4.1 POLYBROMINATED DIPHENYL ETHERS

Concern regarding risks posed by PBDEs is rising, as monitoring programs detect increasing levels of these persistent compounds in almost all environmental samples. In human tissues, an exponential increase of PBDE residues was observed in the past decades [Meironyte *et al.*,1999; She *et al.*, 2002; Sjodin *et al.*, 2003; Schecter *et al.*, 2003; Petreas *et al.*, 2003; Hites, 2004; Sjodin k, 2004]. Entering the key-word “PBDEs” in the National Library of Medicine database (Pubmed), a total of 198 papers have appeared in our survey by November 2004. From that, 77 papers were published in the year of 2004 (until November), reflecting the crescent scientific awareness for this environmental contaminant. However, there is scarce information on PBDE toxicity in animal models, with only a dozen of research groups working in this field. In our survey, we have identified 21 studies out of 198 dealing with experimental data in mammalian systems, including *in vitro* and *in vivo* protocols. This

survey highlights the importance of investigating PBDE effects experimentally, especially *in vivo*.

Data collection about PBDE toxicity thus far suggests that this new environmental compound possesses a wide spectrum of toxicity, affecting various systems (*e.g.* neurobehavioral, reproductive, metabolic and hormonal system) sometimes at low dose levels [Eriksson *et al.*, 2001; Zhou *et al.*, 2001; Meerts *et al.*, 2001; Eriksson *et al.*, 2002; Kodavanti and Derr-Yellin, 2002; Viberg *et al*., 2003a]. However, information collected thus far is insufficient to draw a conclusive picture for risk assessment, creating problems at the regulatory level. Authorities face a dilemma regarding PBDE regulation: on one hand increasing levels detected in human samples claim for instant action, on the other hand, limited amount of experimental data delay the process of hazard identification and risk assessment. This leads to conflicting regulatory approaches among agencies in USA and Europe for regulation of brominated flame retardants. For example, according to the 24th amendment to the marketing and use Directive 76/769/EEC, penta- and octa-BDE (commercial mixtures) were banned for the use in all applications for the European market by August 15th, 2004. In California state (USA), a law signed in 2003 prohibited the use of penta- and octa-BDE by 2008 [Bromine Science and Environmental Forum, 2004]. In contrast to PCBs and dioxins, the hazard of environmental and occupational exposure to PBDEs is unknown, asking for clinical and experimental investigations.

The attempt to understand a few aspects of developmental toxicity of PBDE 99 at low dose range was the main motivation for the present work. The project had an innovatory approach, combining long-term exposure during a sensitive window of development (pre- and postnatal development) with doses close to human exposure. From delivery to adulthood, clear effects were seen in offspring in several endpoints assessed. Furthermore, some results (*e.g.* impairment of rat spermatogenesis at adulthood) suggest that low dose exposure to PBDE 99 causes organizational effects being detected only late in life. These results contribute for human risk assessment of PBDEs, and are unique considering the dose range in which they were observed. Moreover, adverse health effects observed at the dose levels tested in this study contribute to the body of evidence that environmental pollutants are interfering with normal health development. Normally, investigation of low dose effects is neglected due to risk assessment approaches for non-carcinogens that assume a threshold dose, below which there is no risk of harm. However, consistent amounts of data showing

effects below the NOAEL, suggest that the use of a threshold dose is sometimes not suitable for risk assessment of EACs [Calabrese, 2004]. Furthermore, developmental processes involve a complex cascade of events in which minimal external interference may cause permanent deleterious effects. Thus, the assumption of "linearity" in any physiological process is rather reductionist and imprecise, especially when extrapolation factors are used to bridge knowledge gaps.

The characterization of an adverse effect may vary in intensity (*e.g.* from subtle functional effects until malformations or death), leading to different outcomes. Classical toxicological testing for developmental effects focuses on teratology (gross visible damage) and not on subtle outcomes that may be undetectable by physical examination. However, the concept of adverse effects includes any event that affects normal development. In this sense, subtle reproductive impairment or neurological damage may not compromise the viability, but might interfere with reproductive performance and cognitive or motoric abilities of the developing organism. In experimental models, assessment of functional effects is rather considered, but in humans, subtle changes may have profound impact for the society. As an example, we cite the increasing cases of children with ADHD or autism and adults at reproductive age facing problems to conceive (chapter 1). Focusing on functional effects is as important as on those producing gross morphological changes. In that way, the adverse effect concept (gross changes) used in standard testing protocols is also a subject of criticism. For instance, when a NOAEL is established from a teratological study, every functional adverse effect out of the scope of the teratology protocol is ignored. During risk assessment procedures where animal data are extrapolated to human exposure scenarios, functional effects are often not taken into account. In this study, we demonstrated that exposure to a low dose of PBDE 99 caused subtle functional changes in offspring which probably would have been neglected or missed in standard protocols testing acute or chronic toxicity. It is noteworthy that, using a dose level close to environmental exposure, the effects observed experimentally are similar to some features of human disabilities and diseases presented in the first chapter of the thesis. An alternative approach for hazard identification and risk assessment of environmental contaminants would have to include data on functional effects besides the classic toxicological endpoints. The decision to investigate subtle effects must be based on endpoint sensitivity appropriate to the dose level under evaluation.

4.2 THYROID HORMONE EFFECTS

Thyroid hormones are essential to maintain several physiological processes from womb to adulthood. Among them, the role of thyroid hormones during neurological development has attracted special attention in the last years. Balanced levels of thyroid hormones are required for normal brain development; while either excess or deficiency produces significant neurologic impairment [Porterfield, 2000]. Exposure to environmental chemicals has been demonstrated to alter thyroid hormone homeostasis, leading to neurobehavioral deficits. For example, exposing rodents and non-human primates to PCBs during critical periods of development causes learning deficits and hyperactivity at adulthood [Rice, 1999; Berger *et al.*, 2001]. Moreover, thyroid hormone signaling is also essential for Sertoli cell proliferation and differentiation. Hypothyroidism induced by PCBs or PTU may result in an extended proliferation of Sertoli cells, leading to increases in testis size and sperm production at adulthood [Cooke *et al.*, 1994].

In the present study, a low dose of PBDE 99 caused hypothyroxinemia in lactating dams and in offspring after a single administration on gestation day 6. This finding is consistent with other short-term and long-term studies on technical or pure PBDE exposure in rodents [Hallgren *et al.*, 2001; Zhou *et al.*, 2001; Hallgren and Darnerud, 2002]. In dams, the effect of PBDE 99 on thyroid hormone homeostasis was clear at the beginning of lactation and tended to be decreased at weaning, despite no statistic significance (Table 6). On the other hand, T4 levels were markedly decreased in offspring at the end of lactation (Tables 7 and 8). During lactation, high amounts of PBDE pass through the milk, as we have observed high levels of the compound in adipose tissue from dams as well as in offspring at the end of lactation (Table 9 and 10). Therefore the depletion in T4 seen at the end of lactation might be related to the increasing PBDE 99 body burden in offspring. In a pre- and postnatal exposure study, high doses of the BDE technical mixture, DE-71, administered from gestational day 6 to postnatal day 21, was shown to reduce offspring T4 levels at dose levels of 10 and 18 mg/kg and a multiple mechanism of action has been proposed [Zhou *et al.*, 2002]. Increased elimination of thyroid hormones, especially T4, has been proposed to be an important mechanism in PCB induced hypothyroxinemia due to an increased activity of UDPGT, which leads to an accelerated hepatic clearance of T4 [Brouwer *et al.*, 1999]. However, the relationship between serum T4 depletion and induction of the T4-UDPGT activity is not clear in PBDE exposure (demonstrated by three other studies) and does not seem to be an important mechanism in PBDE-induced hypothyroidism [Hallgren *et al.*, 2001; Zhou *et al.*,

2002; Hallgren and Darnerud, 2002]. This is consistent with our findings as we could not see a clear relationship between T4 depression and UDPGT activity in dams and offspring exposed to PBDE 99.

Alternatively, a number of chemicals have been reported to bind to transthyretin (TTR), one of the thyroid hormone-binding transport proteins in plasma of vertebrate species. It is hypothesized that the binding of chemicals to TTR, thereby displacing the natural ligand T4, leads to an increase in clearance of T4 and a decrease in T4 concentrations [Darnerud *et al.*, 1996]. It has been demonstrated that some hydroxylated PBDEs bind with high affinity to human transthyretin *in vitro*. Interestingly, metabolism of PBDE 99 by phenobarbital-induced microsomes (mainly CYP 2B) resulted in 20-60% competition in TTR binding, whereas beta-naphthoflavone-induced microsomes (mainly CYP 1A1) and clofibrate-induced microsomes (mainly CYP 4A3) did not [Meerts *et al.*, 2000]. Another potentially significant property of hydroxylated PBDE metabolites is their ability to bind to the thyroid hormone receptor. Hydroxylated PBDE congeners, 4'-OH-1,3,3',5-tetraBDE and 4'-OH-1,3,3',5',5 –pentaBDE, which theoretically show the highest structural similarity with T3 and T4, respectively, have the highest affinities to the thyroid hormone receptors [Marsh *et al.*, 1998]. In the present study, increasing T4-glucuronidation did not seem to be the mechanism involved in serum T4 depletion. Since TSH levels were also decreased in exposed animals, one can suggest that PBDE 99 might interfere with the hypothalamo-pituitary-thyroid (HPT) axis during a critical period of development.

4.3 ENZYME ACTIVITY

In principle, any phase I or phase II biotransformation enzyme can form proximate (intermediate) or ultimate (reactive species) toxic metabolites that bind to macromolecules and DNA. Phase I oxidation enzymes, however, are the enzymes most frequently involved in the metabolic activation of toxicants. Enzymes of the CYP1A subfamily (1A1, 1A2), for example, are considered as one of the most important groups of monooxygenases that form reactive metabolites from polycyclic aromatic hydrocarbons, aromatic and heterocyclic amines, azobenzene derivatives and planar polyhalogenated biphenyls [Gibson *et al.*, 1994]. On the other hand, phase II reactions involve the conjugation of the xenobiotics to another substance (e.g. glucuronic acid, glutathione, or cysteine) to render it more hydrophilic, facilitating excretion via bile or urine. Modulation of phase I and II activities by an external agent may alter xenobiotic metabolism leading to the production of toxic reactive molecules,

altering pharmacokinetics and diminishing the clearance rate from the organism. Therefore, in toxicological studies biochemical investigation of metabolizing enzyme induction and inhibition is of special interest.

In this study, the administration of a single low dose PBDE 99 on gestation day 6 produced slight changes on hepatic ethohyresorufin-*O*-deethylase (EROD) and uridinediphosphate glucuronosyltransferase (UDPGT) activities during lactation. On PND 22, EROD activity was decreased in dams exposed to PBDE 99 (Figure 8), while no effect was seen in UDPGT activity related to the treatment (Figure 9). A couple of studies from the literature, reporting on the effects of PBDEs on hepatic enzyme activities, support our findings [Carlson, 1980a; von Meyerinck *et al.*, 1990; Hanberg *et al.*, 1991; Hallgren and Darnerud, 2002]. It has been previously reported that PBDEs are able to induce both phase I and phase II xenobiotic metabolism enzymes. CYP1A1 and CYP1A2 were induced, as indicated by increased liver microsomal EROD activity after exposure to a technical mixture, Bromkal 70, in Wistar rats [von Meyerinck *et al.*, 1990] and in H-4-II cells [Hanberg *et al.*, 1991]. Ninety days of exposure to a technical pentabromodiphenyl ether mixture increased *O*-ethyl *O*-p-nitrophenyl phenyl-phosphonothionate (EPN) detoxification and p-nitroanisole demethylation in rats and the elevated levels continued 30-60 days after termination of the exposure [Carlson, 1980a]. However, the interpretation of the effects of technical mixtures is limited due to two confounding factors: firstly, the enzyme induction may be due to impurities present in technical mixtures like polychlorinated dibenzodioxins (PCDD) and dibenzofurans (PCDF) which possess Ah-receptor binding affinity and secondly, the different molecular structure among congeners gives them their own toxicity profile. For example, the congeners 77, 100, 119 and 126 are the PBDEs which exhibit the greatest induction of EROD and also display a relative binding affinity to the Ah receptor, although their maximal EROD activities are less than that of TCDD showing a much higher EC_{50} [Chen *et al.*, 2001; Chen *et al.*, 2003]. On the other hand, the environmental relevant congeners 47 and 99 were not inducers in liver cell lines from rainbow trout (RTL-W1), rat (H4IIE) and human (HepG2) and have a very small relative binding affinity to Ah receptor [Chen *et al.*, 2001; Chen *et al.*, 2003]. In studies investigating phase II enzyme induction, PBDE exposure (either technical mixture or PBDE congeners) resulted in a long-lasting induction of UDPGT activity in rats [Carlson, 1980a; Hallgren and Darnerud, 2002]. Adult Sprague Dawley rats exposed to a high dose of 18 mg PBDE 47 / kg BW for 14 days displayed a 25% increasing UDPGT levels [Hallgren and Darnerud, 2002]. However, a synergistic effect was seen when PBDE 47 was co-administrated with chlorinated paraffins, inducing UDPGT at lower doses [Hallgren and

Darnerud, 2002]. Zhou et al. (2001) also reported that weanling rats exposed to PBDE technical mixtures showed induction of UDPGT activity after 4 days of exposure. However, it seems that PBDEs are not potent UDPGT inducers as significant effects were found only at high dose levels [Zhou *et al.*, 2001; Hallgren *et al.*, 2001; Hallgren and Darnerud, 2002]. In the present study, we report for the first time about modulation of hepatic phase I and phase II enzyme activities mediated by PBDE 99 at such low dose exposure. Although it is difficult to characterize these changes as either "induction" or "inhibition", the differences in EROD and UDPGT activity in dams and offspring may be of biological relevance as the clearance rate and formation of toxic metabolites are unbalanced in exposed animals. Small changes in metabolic clearance can expose the developing organism to high levels of reactive metabolites and /or the xenobiotic itself.

4.4 TISSUE CONCENTRATION

Although the importance of kinetic data for toxicological evaluations has been recognized in the past decades, most reproductive and developmental toxicology studies do not incorporate kinetic endpoints in their protocol design. Pharmacokinetic studies are important for the interpretation of toxicogical experiments; they are particularly useful for the extrapolation of animal experimental data to humans (risk assessment) and for investigation of species differences [Nau H, 1992]. However, the issue becomes critical when the effects of environmental contaminants are evaluated in toxicological protocols. Normally, reproductive and developmental toxicity evaluations do not include kinetic endpoints when environmental contaminants are investigated. This procedure creates uncertainties when human risks posed by these substances are estimated based on animal studies. Furthermore, experimental toxicologists often evaluate doses many orders of magnitude higher than human exposure scenarios, contributing to misinterpretation during risk assessment procedures [Calabrese, 2004]. Therefore, the data on tissue concentration of PBDE 99 presented in this thesis gives a relevant contribution to human risk assessment since a reliable comparison from animal assay to human exposure can be performed.

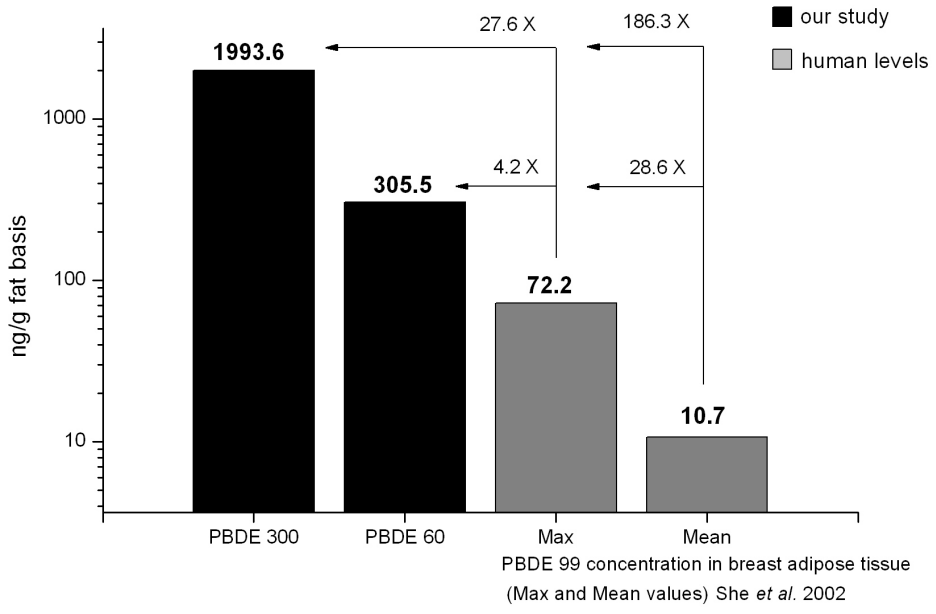
As mentioned above, PBDE 99 possesses a long half-life. Clear indication of continuing exposure after weaning became apparent by significant amounts of the parent compound found in offspring tissue at the end of lactation. This data confirmed our hypothesis that gestational exposure to a single dose of PBDE 99 leads to a long term

exposure. Terminal total body elimination half-life for PBDE 99 in humans was calculated to be 2.9 years as reported by Geyer et al (2004). Additionally, in adult rat adipose tissue the half-life for the same compound was calculated to be 41.6 days in females [Geyer et al., 2004]. In our study, high tissue concentration of PBDE 99 found in dams and offspring at the end of lactation (approximately 37 days after single exposure) corroborates with the data from Geyer et al (2004), indicating that PBDE 99 possesses a long half-life. Another recent study investigating the toxicokinetic of PBDE 47 in mice, demonstrated that this BDE-congener is highly lipophilic and displays long terminal half-life, suggesting a potential for bioaccumulation [Staskal *et al.*, 2004].

It seems that PBDE 99 tissue distribution varies in a dose-dependent manner during lactation. When the percentage of the original dose present in hepatic tissue was calculated, no difference between dose groups (60 and 300 µg/kg) was observed on PND 1 (see chapter 3). However, at the end of lactation (PND 22) a much smaller fraction of the original dose was found in liver from dams exposed to 60µg PBDE 99 (0.024%) than that found in livers of the 300µg PBDE group (0.11%) (chapter 3). A general overview of tissue distribution in adipose tissue and liver can be obtained by calculating the ratio between PBDE 99 levels in both compartments. Through this calculation, we also observed that during lactation the ratios vary according to the dose administered. On PND 1, it seems that PBDE 99 distributes in a

Figure 21: PBDE 99 concentration in rat adipose tissue compared to human concentration levels found in breast adipose tissue.

Human values extracted from: She et al., (2002). Chemosphere, 46(5): 697-707



similar way in both doses tested (ratio: adipose / liver: PBDE 60 = 1.3 and PBDE 300 = 1.7). However, a dose-specific tissue distribution was observed on PND 22, with PBDE 60µg group displaying higher ratios than PBDE 300µg group (ratio: adipose/liver: PBDE 60 = 22.2 and PBDE 300 = 5.3). In another words, PBDE 99 elimination from hepatic tissues is faster in 60µg PBDE 99 group than in animals exposed to 300µg PBDE 99. This "behavior" of PBDE 99 distribution is consistent and had already been observed for other persistent compounds. Abraham et al., (1988) observed that TCDD tissue distribution varies according to the dose, displaying different adipose tissue : liver ratios. Although more detailed investigation should be carried out, it seems that the elimination of PBDE 99 through breast milk interferes with the tissue distribution in a dose-dependent way.

In this thesis, the most important analysis drawn from PBDE 99 tissue concentration relies on the possibility to compare the dose levels used experimentally with human exposure scenarios. Although higher PBDE concentrations have been reported in human tissues [Schecter *et al.*, 2003; Hites, 2004], we decided to use the PBDE 99 concentrations found in human breast adipose tissue by She et al (2002). For this purpose, dam's adipose tissue concentration on PND 1 (Table 9) was plotted together with the mean and maximum PBDE 99 levels reported by She et al., 2002 (Figure 21). Compared to human data, the dose level employed in the present study is about 29-fold and 186-fold higher than the mean PBDE 99 concentration found in human breast adipose tissue (Figure 21). If we take into account the population bearing the highest level of this congener, we are only 4-fold and 28-fold higher than human exposure levels. To date, no other research group has reported an *in vivo* effect so close to human exposure levels.

4.5 NEUROBEHAVIOR DEVELOPMENT

Developmental exposure to chemicals may lead to a range of functional disturbances in the offspring. A number of chemicals are known to produce developmental neurotoxic effects in humans and other species. For example, lead affects brain development and effects on fertility, metabolism and development as a whole have been observed [Gidlow, 2004]. Because of the established role of thyroid hormones in neural development (*e.g.* neurogenesis, migration of cells in the central nervous system and cell differentiation), substances that interfere with thyroid hormone homeostasis are assumed to exert neurobehavioral effects [Porterfield, 2000]. Moreover, several environmental compounds have the ability to produce neurotoxicity independently from the thyroid hormone signaling

system. They can act directly in the developing brain altering other signaling cascades essential for normal development impairing neuronal proliferation and differentiation of specific nerve cells. Increases in the prevalence of neurodevelopmental disorders over the past 30 years make it imperative to reverse this trend (chapter 1). Because this trend could be partly the result of exposure to environmental contaminants, it is also imperative to prevent further exposure to synthetic chemicals that are under suspect.

Assessment of physical development in offspring (*e.g.* eye opening, fur development, eruption of incisors) and postnatal reflexes are validated endpoints employed in neurotoxicity and postnatal studies [Organisation for Economic Co-operation and Development, 2004]. These observations often show "when" rather than "if", the various landmarks first appear and are used to assess delayed or accelerated developmental time courses for the specific parameters being studied [Lochry *et al.*, 1986]. According to the Organisation for Economic Co-operation and Development (OECD) guidelines, the registration of ear unfolding, eruption of incisor and eye opening should be considered when appropriated in postnatal studies [Organisation for Economic Co-operation and Development, 2004]. The same guidelines recommend the evaluation of postnatal reflexes in neurotoxicity and postnatal studies. In the present study, offspring exposed to a low dose of PBDE 99 or PTU displayed changes in the postnatal reflexes and in the developmental landmarks investigated. Although the assessment of such endpoints does not give us mechanistic interpretation, general effects seen in the time of eruption of incisors, eye opening and cliff-drop aversion reflex suggest that the exposure to PBDE 99 or PTU may affect sensory-motor development.

Developmental exposure to PBDE 99 also leads to long-lasting behavioral effects in offspring which persist at least until puberty. We observed hyperactivity in weanling rats exposed to 300 µg PBDE 99 or PTU. At puberty, the effect on PTU animals was no longer apparent, but both PBDE 60 and PBDE 300 offspring were still more active than controls. Other investigators have found neurodevelopmental disturbances when rodents were exposed to PBDEs [Eriksson *et al.*, 2001; Branchi *et al.*, 2002; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b; Branchi *et al.*, 2003]. NMRI mice exposed to a single dose of decabrominated diphenyl ether (PBDE 209) on postnatal day 3 showed a permanent increase in activity that worsened with age [Viberg *et al.*, 2003b]. The same group also reported that a single dose of PBDE 153 on PND 10, caused changes in spontaneous behavior (hyperactivity), impaired learning and memory and reduced the amounts of nicotinic receptors in the hippocampus, through alpha-bungarotoxin assay [Viberg *et al.* 2003a]. Previously, Viberg *et al.* (2002) have also reported that neonatal exposure to PBDE 99 altered the cholinergic transmitter system in

adult mice, as they observed changes in the response of nicotine-induced behavior [Viberg *et al.*, 2002]. Supporting our data, Eriksson et al. (2001) reported that neonatal exposure (single dose on postnatal day 10) to PBDE 99 or 47 disrupts spontaneous behavior in mice and increases activity levels in a dose-response fashion which appear to be permanent and to worsen with age [Eriksson *et al.* 2001]. In his study, the locomotor activity was measured in three different sessions of 20-min each (0-20 min, 20-40 min. and 40-60 min.) in 2- and 4-month old NMRI mice. Animals exposed to PBDE 99 and 47 were transiently hypoactive in the first 20 minutes of habituation, which was reversed at the last 20 minute period of measurement being more active than the control. Similar to our findings, Eriksson *et al.* also reported that the effects were persistent as the animals were still hyperactive at approximately 110 days after exposure. The literature indicates that neurobehavior development is the most sensitive system to PBDE-induced toxicity reported thus far. Using a different method to assess rat locomotion, we found similar effects to those reported by Eriksson *et al., i.e.* offspring were hyperactive. However, the advantage of the Mobilitron® device (reported previously in the section 3.2.2) allowed us to perform measurements in an environment similar to animal's housing conditions. In that way, we diminished the influence of a "novel environment" which normally leads to exploratory behavior rather than measurement of basal locomotor activity [Mead *et al.*, 1996]. Moreover, the recording time of each measurement was much longer than that reported by Eriksson *et al.* (our study: 24 hours *vs.* Eriksson study: 60 min) and the large number of animals evaluated in this study increases the statistical power and sensitivity to detect minor changes. The hyperactivity induced by the low dose of PBDE 99 may be translated into changes in offspring basal locomotor activity, similar to ADHD in human.

The crucial rule of thyroid hormones (TR) during brain development is well known. TR increases the rate of neuronal proliferation in the cerebellum, they are involved in the pattern of neuronal migration and act as the "time clock" to end neuronal proliferation and stimulate differentiation [Porterfield, 1994]. Therefore, any disturbance in thyroid hormone homeostasis (e.g. PTU exposure) can cause serious impairment of the neurological development [Porterfield, 1994]. Previous studies support our findings as they report hyperactivity in rodent offspring after pre- and postnatal hypothyroidism induced by goitrogens such as PTU [Davenport *et al.*, 1976; Tamasy *et al.*, 1986; Akaike *et al.*, 1991; Goldey *et al.*, 1995]. However, the behavioral changes in the PBDE 99 groups (persistent at least until PND 71) observed in this study are not similar to our reference group for thyroid hormone-mediated effects (PTU) (transient, hyperactivity only on PND 36), suggesting that

the neurotoxicity-induced by PBDE 99 may stem from different mechanisms. Even though more mechanistic studies are lacking, the cholinergic system seems to be affected after neonatal exposure to PBDE as Viberg *et al.* (2003a) found reduced amounts of nicotinic receptors in the hippocampus of exposed animals using an alpha-bungarotoxin assay. Furthermore, the response of the cholinergic agent nicotine was altered in mice neonatally exposed to PBDE 99 [Viberg *et al.* 2002; Viberg *et al.*, 2003b]. Therefore, hyperactivity induced by PBDE 99 might be explained by changes in the cholinergic system during pre- and postnatal exposure. Nevertheless, one should not rule out other mechanisms as some hydroxylated PBDE metabolites have been shown to possess high binding affinities to the thyroid hormone receptor [Marsh *et al.,* 1998]. It is plausible that also the binding of the PBDE 99 molecule or its metabolite to the thyroid hormone receptor in the developing brain could cause neurobehavioral disturbance in the offspring.

4.6 MALE REPRODUCTIVE SYSTEM

The issue of persistent chemical contamination normally focuses on bioaccumulation, neurotoxicity and carcinogenicity of different compounds. However, the interference of chemical exposure with reproductive capability is becoming a major concern in toxicological investigations. The male reproductive system has been shown to be a very sensitive endpoint when the insult occurs during critical periods of development [Dalsenter *et al.*, 1997; Faqi *et al.*, 1997; Faqi *et al.*, 1998; Andrade *et al.*, 2002; Kuriyama and Chahoud, 2004]. Moreover, human epidemiological data support the investigation of possible reproductive effects mediated by environmental contaminants.

Developmental exposure to PBDE 99 doses resembling human exposure levels causes impairment of male reproductive health in adult offspring. Subtle changes in testis and epididymis weights, associated with impairment of spermatogenesis were observed in rats prenatally exposed to PBDE 99. Although no clinical signs of toxicity were seen during gestation/lactation, the administration of a single low dose PBDE 99 on gestation day 6 caused permanent alterations in the reproductive system of adult male offspring. This is the first report on effects of PBDE 99 on male reproductive performance as our search in the literature failed to produce similar data. In rat models, endpoints like sperm count, daily sperm production, sexual hormones and sexual behavior are more sensitive than fertility studies (the capacity of exposed males to fertilize and sire normal offspring) since the number of sperm enormously exceeds the number necessary to ensure reproductive competence

[Aafjes *et al.*, 1980; Faqi *et al.*, 1997]. In the present study, the impairment of normal spermatogenesis did not produce gross changes in the capability of exposed males to sire a normal outcome, except for animals exposed to PTU and PBDE 60, which required more time to impregnate untreated female rats (Figure 19). However, in humans relatively small changes in sperm production may have severe consequences for human reproduction [Zenick *et al.*, 1989]. Our results are in consistency with previous investigations that reported impairment of spermatogenesis after prenatal exposure to other persistent compounds. Pre- and postnatal exposure to TCDD, lindane and PCB 118 has been demonstrated to impair spermatogenesis and disturb sexual behavior following decreases in serum testosterone levels in rats [Faqi *et al.*, 1998; Dalsenter *et al.*, 1997; Kuriyama and Chahoud, 2004]. In our study, sexual behavior of male offspring was normal although fewer animals from the PBDE 300 group had two ejaculations or more after 20 minutes. In our rat model, exposure to low dose PBDE 99 during development affects rat spermatogenesis (with slight changes in reproductive organ weights), and minor changes were observed in endpoints like sexual behavior and fertility studies.

The growth and maturation of the developing testis as well as the maintenance of spermatogenesis are regulated by several endocrine and paracrine factors. Among them, thyroid function during early life has a major impact on regulating testicular growth and function. When rats were made hypothyroid during a critical window of neonatal development, permanent increases in adult testis size and sperm production have been observed [Cooke *et al.*, 1992]. However, this effect occurs only when rats are made hypothyroid during the first week of postnatal development [Cooke *et al.*, 1992]. Using a dose 200-fold lower than that reported by Cooke et al. (1992), we observed that prenatal hypothyroidism induced by PTU caused an opposite effect namely decreased sperm production and testis size. Impaired spermatogenesis and reduced testicular weight seen in males exposed to both doses of PBDE 99 might also be correlated to alterations in thyroid hormone concentrations. However, the mechanisms underlying these effects remain to be elucidated. Another hypothesis that may explain the decreased spermatogenesis is a possible interference of PBDE 99 with FSH and/or testosterone (TT) levels. The homeostasis of FSH and TT is important for quantitatively normal spermatogenesis. During development, male sex organs are fully imprinted within 10-20 days after birth [Amann, 1986]. Imprinting requires basal levels of testosterone (or its metabolites) secreted by the testis in order to warrant the responsiveness of these organs to steroids [Wilson *et al.*, 1981]. We found no differences in serum testosterone levels in adult offspring, and FSH was not measured.

However, we can not rule out the possibility of early depression of FSH and testosterone in those rats during a critical phase of development which might affect the spermatogenesis at adulthood.

Testis size and sperm production are directly correlated to the Sertoli cell number. Since we observed a decrease in testis size followed by impaired spermatogenesis, we also consider the possibility of a permanent injury caused by PBDE 99 on Sertoli cell proliferation and /or maturation. Moreover, the integrity of seminiferous tubule is also correlated with healthy spermatogenesis. To investigate a possible effect of PBDE 99 on Sertoli cell number and of seminiferous tubule integrity, testicular morphometric analysis was performed in exposed rats. As depicted in chapter 3, no effect related to the treatment was seen after analysis. Sertoli cell number and morphometric scores of seminiferous tubule were all within the normal range (Table 14). However, at the functional level we could not determine whether the seminiferous epithelium is normal, since morphometric analysis gives us only morphological but not functional evaluation. Thus, the mechanism underlying the impairment of spermatogenesis induced by PBDE 99 might be different than reducing the Sertoli cell number.

Alternatively, flow cytometry method has been shown to be a useful method which provides quantitative evaluation of different cell types on the basis of their DNA ploidy/stainability level. Furthermore, this analysis gave us a rough qualitative evaluation of impaired spermatogenesis. With flow cytometric analysis a statistically significant decrease in the ratio of haploid / diploid cells was observed in the PBDE 99 group (Figure 18), which corroborates the reduced number of spermatid/sperm counts. Although we did not observe differences in the number of mature haploid cell (mostly elongated spermatids) among the groups (Figure 17), the slight decrease in immature haploid (round/elongated spermatids) and increase in the diploid population observed in PTU and PBDE exposed animals suggest an impairment of normal spermatogenesis. Not only the classical method of sperm counting but also flow cytometry analysis indicate that exposure to a low dose of PBDE 99 during development permanently affects adult male spermatogenesis. However, it seems that the severity of sperm/spermatid count reductions seen in treated animals (between 15% to 35%) does not reflect the moderate changes in the flow cytometric analysis. Flow cytometry offers advantages in terms of objectivity, rapidity and analysis of a large number of cells [Suter *et al.*, 1998b; Suter *et al.*, 1998a; Yoon *et al.*, 2001]. However, the sensitivity of this method is sufficient to detect massive testicular damage but is not suited for the detection of limited but

significant toxic effects [Suter *et al.*, 1997], which may explain the moderate effects seen in the flow cytometry analysis.

Recently, evidence from epidemiological studies suggests that the observed decline in human semen quality, male fertility and increase in testicular cancer in the last 50 years are associated with environmental contaminant levels [Rozati *et al.*, 2002; Dallinga *et al.*, 2002; Hauser *et al.*, 2002]. Knowing that human sperm count is near the threshold for the number of sperm needed to ensure reproductive competence, one should also considerer that small fluctuations in human spermatogenesis due to exposures to environmental pollutants might also interfere with normal reproductive capacity. The exposure to a low dose of PBDE 99, which resembles the human exposure levels, caused permanent impairment of spermatogenesis in rats.

4.7 CONCLUDING REMARKS

Tracing a parallel between increasing prevalence of human health diseases (e.g. neurodevelopmental disorders) with data on the growth in synthetic chemical production, the data begin to merge around 1970. At approximately the same time, the first generation of humans exposed to synthetic chemicals on a large scale in the womb began to have children (Table 16). Although individuals were being exposed since the early 1920s, it was not until the end of World War II that exposure increased to such an extent that daily exposure led to accumulation of chemical residues in the body [Colborn, 2004]. A generation analysis shows that around 1950s surged the first generation of offspring exposed in the womb to high levels of different chemicals (Table 16). Interestingly, increasing incidence of rare neurodevelopmental disorders appeared by the 1970s, the same time that post-World War II individuals were having their own babies [Colborn, 2004]. In chapter 1, the body of evidence indicating that environmental exposure to several chemicals is interfering with human health is presented. However, a conclusive association between human health problems and environmental pollutants is very difficult to draw due to several variables like: individual variability, mixed-type of exposure, antagonistic and synergistic effects. In this context, experimental investigations focusing on mode of action and mechanism of toxicity are

Table 16: Chronology of human exposure

Years	Exposure Scenario
1920s-1930s	BPA, PCBs, and DDT commercially introduced. Chlorine industry expanding. Discrete postnatal and prenatal exposure
1940s-WWII	First wide-scale production and exposure to the above and other chemicals including plastics and chlorinated compounds as technology advanced
1940s-1950s	First generation widely exposed postnatally and some who may have been exposed prenatally.
1950s-1970s	First generation born that was widely exposed prenatally.
1970s-1990s	First generation that was widely exposed prenatally reached reproductive age.
1980s-present	Second generation born that was exposed in the womb and beginning to produce the third generation. Production volume and exposure still increasing.

* extracted from: Colborn (2004). Neurodeveolpment and Endocrine Disruption. EHP, 11(9): 944-949
WWII – World War II

important for an accurate assessment. Studies investigating single compounds with high degree of purity have been encouraged so far [Brouwer *et al.*, 1999; Schantz *et al.*, 2003]. Except for few compounds like PCBs, dioxins, lead and mercury, great experimental effort is still to be made to assess the risks posed by other environmental compounds like PBDEs, perchlorates and phthalates. Experimental investigation of co-administration of different compounds would give us an approximate picture of human exposure scenarios. However, variable dose-related effects demonstrated by several EACs create problems to accurately design experimental protocols investigating more than one compound. In this context, the set of data presented in this thesis contributes to the knowledge of toxic effects posed by the brominated flame retardant PBDE 99. Using a rat model, we found effects in a dose range close to human exposure. Considering that PBDE 99 levels in human body burden co-exist with many other environmental pollutants, efforts on investigation of synergistic, additive and antagonistic effects should be made for human risk assessment.

Exposing offspring from early embryonic stages until the end of lactation to low doses of PBDE 99 causes multiple adverse effects which seem to be permanent, as they were apparent until adulthood. The data presented here are far from being conclusive but highlights the importance of investigating the effects of environmental pollutants at low dose levels close to human exposure. During organizational stages of development (gestation and lactation periods), responses to endocrine disruption are unlike the typical responses in adulthood. Consequently, testing with mature animals misses the organizational damage from pre- and postnatal exposures. In addition, most traditional toxicological tests use doses 1000-1000,000 fold of the equivalent physiological range at which the endocrine systems operate and well above realistic exposure concentrations to synthetic chemicals. The two doses investigated in this study are the lowest doses of PBDE 99 to cause an *in vivo* effect in experimental model reported so far.