Developmental exposure to PBDE 99 and PCB affects estrogen sensitivity of target genes in rat brain regions and female sexual behavior

Walter Lichtensteiger[1], Oliver Faass[1], Raffaella Ceccatelli[1], Margret Schlumpf[1]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Zurich

Introduction

We recently reported effects of PBDE99 (2,2',4,4'5-pentabromoBDE) on sexual differentiation processes in rat reproductive organs and central nervous system[1]. These studies were prompted by reports on an increase of PBDE levels in human milk, an indicator of the body burden of pregnant women and of potential exposure of the nursing infant, during the last decade[2]. Even higher human adipose tissue and milk levels were reported for North America[3-6]. PBDE99 is present in human and animal samples and exhibits developmental neurotoxicity in mice[7]. The developing brain is subject to the organizing action of estradiol locally formed from circulating testosterone, and thus represents a target for endocrine active chemicals. One molecular mechanism by which chemicals may interfere with sexual brain differentiation, may be a change in the expression of sex hormone (estrogen)-regulated genes. Such effects may manifest themselves in mRNA expression levels, or in the sensitivity of the genes to estrogen. In order to detect alterations of the latter, more subtle parameter, we have conducted experiments in developmentally chemical-exposed rat offspring that were gonadectomized in adulthood and injected with a challenge dose of estradiol. Effects of PBDE99 were compared with those of a commercial PCB mixture, Aroclor 1254, which had previously been found to influence sexual brain differentiation[8]. We analyzed the expression of estrogen-regulated genes in ventromedial hypothalamus (VMH) and medial preoptic area (MPO), two brain regions that are part of a network involved in the integration of environmental cues, sexual behavior and gonadal function[9]. Since prominent changes were observed in VMH which is particularly important for female sexual behavior, the study was completed by a behavioral analysis.

Methods and Materials

Animal experimentation. The study was conducted on Long Evans rats (Møllegard, Denmark) housed under controlled conditions (lights on 02.00-16.00, 22 ± 1 °C). Time-pregnant females were given one daily subcutaneous injection of chemical dissolved in olive oil or of vehicle from gestational day (GD)10 to GD18 (9 injections) (GD1=24 hr after onset of mating period). Experimental groups included: PBDE99 (2,2',4,4',5-pentabromoBDE, Promochem GmbH, Wesel, Germany, purity > 99%), 1.0 mg/kg/day (P1) or 10 mg/kg/day (P10); the commercial PCB mixture, Aroclor 1254 (Promochem, Wesel), 10 mg/kg/day (Aro10), and vehicle controls (olive oil)

accompanying each treatment group. Pregnancy and postnatal developmental landmarks were monitored as previously described[1]. Litter size was adjusted to 8-10 pups on postnatal day (PN) 2 (PN 1 = day of birth). At 70 days of age, part of the male and female offspring out of different litters were gonadectomized in general anesthesia. After a recovery period of two weeks, at 84 days of age, these animals were injected subcutaneously with a single dose of estradiol (10 µg/kg in olive oil) or with vehicle (vehicle controls), and sacrificed 6 hours later (estrogen challenge). Additional offspring were studied intact at 84 days or 4 months of age[1]. At these time-points, the brain was excized and stored at -80°C, uterus, ventral prostate and dorsal (dorsal + lateral prostate were weighed and stored in liquid nitrogen. Two brain pieces, medial preoptic area and ventromedial hypothalamus (with ventromedial hypothalamic nucleus), were dissected from 100 µm frontal cryostat sections. This paper will be limited to brain data.

mRNA analysis. mRNAs encoding for estrogen receptor (ER) alpha, ER beta, progesterone receptor (PR), preproenkephalin (PPE), and cyclophilin were determined by Real Time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland), and the TaqMan universal master mix (Applied Biosystems), as described[10]. mRNA levels of target genes were normalized to cyclophilin as reference gene.

Behavioral analysis. Female sexual behavior was studied in offspring of the 10 mg/kg PBDE99 (P10) and control groups (age 4-5 months). At the onset of the dark period (16.00 hr), female offspring with estrus confirmed in the morning (vaginal smear) were placed into an arena illuminated with red light, and mated with a normal, untreated male. Sexual behavior was monitored by video camera during 30 min in the absence of personnel.

Statistics. mRNA data of different experimental groups were compared by one-way ANOVA followed by pairwise comparisons with Bonferroni correction, differences between P10 and Control group for individual behavioral parameters by unpaired t-test with Welch correction.

Results and Discussion

Expression of mRNAs encoding for PR and PPE, the precursor of the neuropeptide enkephalin, in ventromedial hypothalamus (VMH) and medial preoptic area (MPO) is linked to the control of sexual behavior and gonadal function (see below). We previously reported on PR mRNA in MPO and VMH of rat offspring exposed to PBDE 99 or Aroclor 1254 during development and analyzed in adulthood without further experimental manipulation ("steady state" condition)[1]. Prenatal treatment with both compounds was followed by a significant decrease of PR mRNA in female VMH, resulting in a loss of the physiological sex difference of PR mRNA (higher in females). PPE mRNA levels were also affected (Tab. 1). Additional changes were observed for ER alpha and ER beta (ref. 1, O. Faass et al., in preparation).

Table 1. Synopsis of significant changes in levels of PR mRNA and PPE mRNA in brain regions of adult rat offspring after prenatal treatment with PBDE 99 or Aroclor 1254

Region	mRNA	Sex	PBDE 99		Aroclor 1254
			1 mg/kg	10 mg/kg	10 mg/kg
VMH	PR mRNA	Male	Increased	≈	≈
		Female	Decreased	Decreased	Decreased
	PPE mRNA	Male	Increased	≈	Decreased
		Female	Increased	≈	≈
MPO	PR mRNA	Male	≈	≈	≈
		Female	≈	≈	≈
	PPE mRNA	Male	Decreased	≈	≈
		Female	≈	≈	≈

VMH: Ventromedial hypothalamus, MPO: medial preoptic area.
≈: No significant change ($p > 0.05$), ANOVA, pairwise comparisons (Bonferroni).
Data from Lichtensteiger et al., 2003, and O. Faass et al., in preparation.

The interplay between peripheral endocrine glands and the brain depends on the responsiveness of neural centers to estrogen. Changes in the sensitivity for estrogen may impair the functional integrity of neuroendocrine regulation. In order to assess the acute responsiveness of target genes to natural estrogens, we injected adult gonadectomized rat offspring with estradiol (10µg/kg s.c.) and studied mRNA levels 6 hours later (Fig. 1). In controls, estradiol induced PR mRNA expression in both brain regions. In VMH, the induction of PR mRNA by estradiol was significantly higher in females. Both doses of PBDE 99 as well as Aroclor completely abolished this response in MPO. An analogous but somewhat weaker effect was also observed in female VMH. Male VMH exhibited a more complex pattern with a suppression of estrogen-induced PR mRNA induction by 1 mg/kg PBDE 99 and Aroclor, and an enhanced induction by 10 mg/kg PBDE 99. Changes in responsiveness to estradiol were also observed for PPE mRNA.

In female rats, the display of sexual behavior is facilitated by the sequential action of estrogen followed by progesterone in combination with estrogen. Under the influence of estrogen, PR mRNA and PPE mRNA are induced in VMH[11-13]. Female sexual behavior depends on the up-regulation of the two mRNA species; the behavior can be blocked by local injection of antisense oligonucleotides against either PR mRNA or PPE mRNA[14,15]. In view of the importance of VMH for female sexual behavior, we tested sexual behavior in adult female offspring developmentally exposed to 10 mg/kg PBDE99. Exposed females exhibited a massive impairment of sexual behavior, as illustrated in Fig. 2 by the effect on lordosis quotient (number of lordosis reactions/number of matings) and on incentive behavior (jump and wiggle) displayed by the female to attract the male. Feminization of a non-reproductive sexually dimorphic behavior (sweet preference) in male rats has been observed after developmental exposure to a reconstituted PCB mixture composed according to the congener pattern in human breast milk, however, Aroclor 1254 was not effective in that experiment[8]. At the gene expression level, effect patterns of PBDE99 and

the commercial PCB mixture, Aroclor 1254, also differ in part, both in brain and reproductive organs[1].

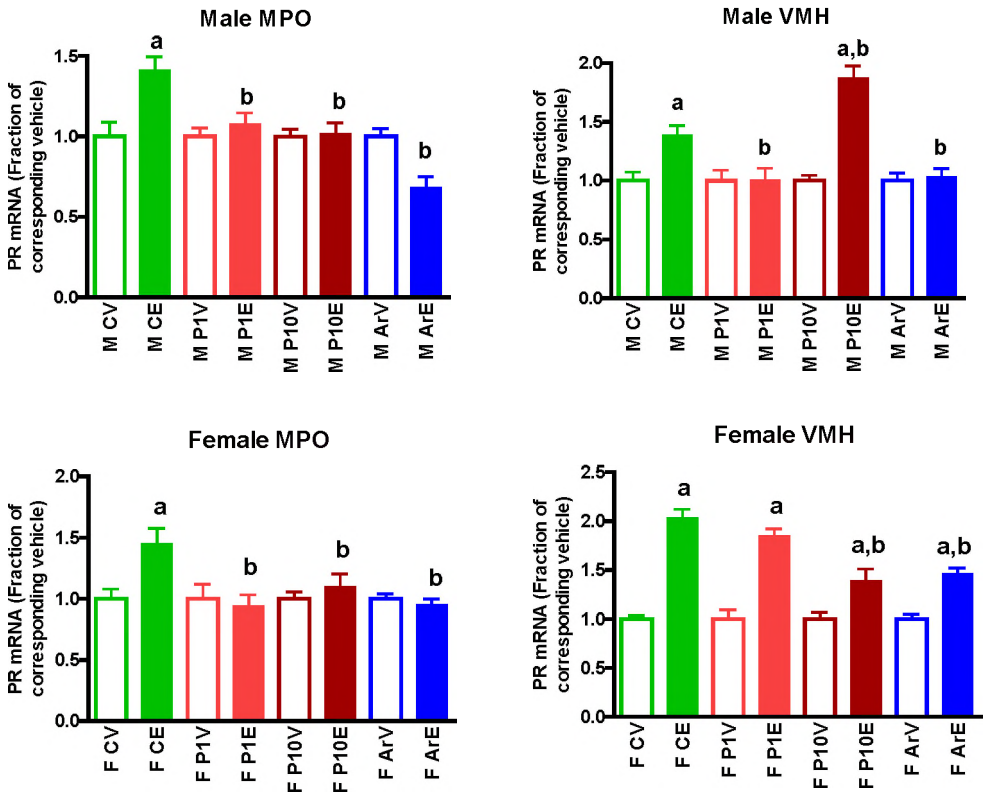

Fig. 1. Progesterone receptor (PR) mRNA levels in medial preoptic area (MPO) and ventromedial hypothalamus (VMH) of adult gonadectomized rat offspring 6 hours after s.c. injection of estradiol (10µg/kg). Mean ± SEM (n=6-9) relative to the mean of the corresponding vehicle-injected group. F: Females, M: Males. Prenatal treatment: C: Control, P1: PBDE 99 1 mg/kg, P10: PBDE 99 10 mg/kg, Ar: Aroclor 1254 10 mg/kg. Adult treatment: V: Vehicle, E: estradiol. a = different from corresponding vehicle-group $p < 0.05$ to 0.001; b = different from estradiol-injected control group (FCE, MCE) $p < 0.05$ to 0.001. Sex difference in estrogen effect in VMH of controls $p < 0.001$.

The PBDE-NTOX project was designed for hazard identification rather than for risk assessment. Nevertheless, it is interesting to compare the limited information on rat tissue levels with epidemiological data in humans and wildlife. Treatment with 1 or 10 mg/kg resulted in the following PBDE99 levels in rat offspring (analyzed by EUKOS, Plön, Germany): Neonatal brain 0.04 ± 0.02 µg/g lipid (n=2), and 0.21 ± 0.006µg/g lipid (n=2) (pooled brains). Adipose tissue of

120 day-old adult offspring 0.42 ± 0.14 µg/g lipid (n=2), and 7.4 ± 3.0 µg/g lipid (n = 11) (males + females combined). For North America, mean human adipose tissue and milk levels of PBDE99 range between 0.01 and 0.03µg/g lipid, with upper levels up to 0.11µg/g lipid[3,5,6], while European levels are considerably lower[6]. For Californian harbor seal blubber, a mean PBDE99 concentration of 0.112µg/g lipid was reported, with a range up to 0.303µg/g lipid[3]. Thus, the tissue levels of the lower dose of PBDE99 come comparatively close to the upper concentration range of PBDEs reported for humans and marine mammals in North America.

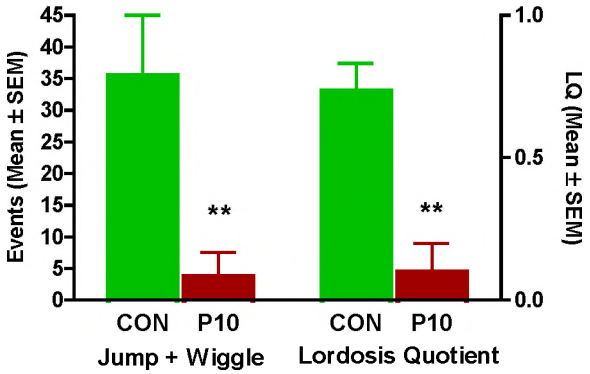

Fig. 2. Female sexual behavior in adult rat offspring following developmental exposure to PBDE99 (10 mg/kg). Mean ± SEM (n = 9) of number of jumping or wiggling events and number of lordosis reactions/number of matings (lordosis quotient) during 30 min observation time. ** different from control $p < 0.001$ for lordosis quotient, $p = 0.01$ for jump + wiggle.

In conclusion, our data demonstrate that exposure to PBDE99 during pre- and postnatal development can interfere with expression and estrogen sensitivity of sex hormone-regulated genes in sexually dimorphic brain regions, and with female sexual behavior. The developing neuroendocrine brain appears to represent a sensitive target for endocrine disruptors, and should receive more attention besides peripheral endocrine and reproductive organs.

Acknowledgements
The investigation was supported by the 5th EU Framework Programme (PBDE-NTOX) and the Swiss Environmental Protection Agency (BUWAL).

References

1. Lichtensteiger, W., Ceccatelli, R., Faass, O., Ma, R., and Schlumpf, M. (2003). In: Organohalogen Compounds, Vol. 61, G. Hunt, R. Clement, Eds., RPJ Ass., Natick, USA, p. 84.
2. Meironyte, D., Noren, K., Bergman, A.; (1999) J. Toxicol. Environ. Health Part A, 58, 329.
3. She J., Petreas M., Winkler J., Visita P., McKinney M. and Kopec D. (2002). Chemosphere 46, 697.
4. Petreas M., She J., Brown F.R., Winkler J., Windham G., Rogers E., Zhao G., Bhatia R. and Charles M.J. (2003). Environ. Health Perspect. 111, 1175.
5. Schecter A., Pavuk M., Päpke O., Ryan J.J., Birnbaum L. and Rosen R. (2003). Environ. Health Perspect. 111, 1723.
6. Sjödin A., Patterson D.G. Jr. and Bergman A. (2003). Environ. International 29, 829.
7. Eriksson, P., Jakobsson, E., Fredriksson, A.; (2001) Environ. Health Perspect., 109, 903.
8. Hany J., Lilienthal H., Sarasin A., Roth-Härer A., Fastabend A., Dunemann L., Lichtensteiger W. and Winneke G. (1999) Toxicol. Appl. Pharmacol. 158, 231.
9. Segovia S. and Guillamon A. (1993). Brain Res. Rev.18, 51.
10. Maerkel K., Lichtensteiger W., Durrer S. and Schlumpf M. Environ. Toxicol. Pharmacol. in press.
11. Brown T.J., Clark A.S. and MacLusky N.J. (1987). J. Neurosci. 7, 2529.
12. Lauber A.H., Romano G.J., Mobbs C.V., Howells R.D. and Pfaff D.W. (1990). Mol. Brain Res. 8, 47.
13. Lauber A.H., Romano G.J. and Pfaff D.W. (1991). Neuroendocrinology 53, 608.
14. Ogawa S., Olazàbal U.E., Parhar I.S. and Pfaff, D.W. (1994). J. Neurosci. 14, 1766.
15. Nicot A., Ogawa S., Berman Y., Carr K.D. and Pfaff D.W. (1997). Brain Res. 777, 60.