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Prenatal Aroclor 1254 exposure and brain sexual differentiation: Effect on the expression of testosterone metabolizing enzymes and androgen receptors in the hypothalamus of male and female rats

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Abstract

Polychlorinated biphenyls (PCBs) are industrial pollutants detected in human milk, serum and tissues. They readily cross the placenta to accumulate in fetal tissues, particularly the brain. These compounds affect normal brain sexual differentiation by mechanisms that are incompletely understood. The aim of this study was to verify whether a technical mixture of PCBs (Aroclor 1254) would interfere with the normal pattern of expression of hypothalamic aromatase and 5-alpha reductase(s), the two main enzymatic pathways involved in testosterone activation and of androgen receptor (AR). Aroclor 1254 was administered to pregnant rats at a daily dose of 25 mg/kg by gavage from days 15 to 19 of gestation (GD15–19). At GD20 the expression of aromatase, 5-alpha reductase types 1 and 2 and androgen receptor (AR) and aromatase activity were evaluated in the hypothalamus of male and female embryos. The direct effect of Aroclor was also evaluated on aromatase activity adding the PCB mixture to hypothalamic homogenates or to primary hypothalamic neuronal cultures. The data indicate that aromatase expression and activity is not altered by prenatal PCB treatment; 5-alpha reductase type 1 is similarly unaffected while 5-alpha reductase type 2 is markedly stimulated by the PCB exposure in females. Aroclor also decreases the expression of the AR in females. The observed *in vivo* effects are indicative of a possible adverse effect of PCBs on the important metabolic pathways by which testosterone produces its brain effects. In particular the changes of 5-alpha reductase type 2 and AR in females might be one of the mechanisms by which Aroclor exposure during fetal development affects adult sexual behavior in female rats.

Keywords: Aromatase; 5-Alpha reductase types 1 and 2; Fetal brain; Rat; Aroclor 1254; PCBs; Androgen receptor

1. Introduction

Brain sexual differentiation represents a complex series of events producing morphological as well as physiological differences in the brain of male and female animals. Recent evidence indicates that chromosomal differences between sexes and social/environmental conditions might affect brain sexual differentiation [1,2], however, it is generally recognized that the hormonal "milieu" is the leading force controlling this process at least in rodents. In these animals the pre- and perinatal androgen secretion by testes is the most important event leading to peripheral and central sexual dimorphism.

It is likely that testosterone (T) does not masculinize the brain acting only in its native configuration, but it mostly needs local conversion into active metabolites. The two main enzymatic pathways involved in T activation are: aromatase, which converts T into estradiol, and 5-alpha reductase, which transforms T into the more potent androgen dihydrotestosterone (DHT). It is well known that, at least in rodents, estradiol derived from intracellular T aromatization is the major effector of brain masculinization [3]. The aromatization process occurs only in neurons because the glial compartment is devoid normally of the enzyme [4]. During development, aromatase mRNA expression and the enzymatic activity show, at least in the rat preoptic/hypothalamic area, an apparently specific pattern. It has been reported that both aromatase mRNA and activity, which are low on gestational days (GD) 15–16, increase to peak on GD19–20 and then gradually decrease during the perinatal period, reaching low levels observed in adulthood [5–7]. Furthermore, it is generally agreed that the enzyme levels are higher in the male than in the female brain [7–11].

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Two 5-alpha reductases isozymes (5-alpha-R1 and 5-alpha-R2) encoded by different genes and with distinct physiological functions are involved in DHT formation. 5-Alpha-R1 has a low affinity for T and other three cheto-delta four steroids and is expressed in tissues of both sexes (with the highest levels in the liver) including brain (neurons and glia) [12]. This isoform is thought to be constitutive and mainly catabolic. On the other hand, 5-alpha-R2, the isozyme involved in the activation of T in the peripheral male androgen-dependent structures, is localized in neurons and expression peaks in the rat brain during the perinatal period in parallel with the rise of T secretion [13]. This isoform might be involved, together with aromatase, in directing some aspect of brain differentiation [14].

Development of the sexual dimorphic morphological and functional characteristics of the mammalian brain during pre- and perinatal development appears to be stage-dependent. A major effect is exerted by the levels of the steroid metabolizing enzymes present in the brain.

Emerging evidence indicates an adverse influence of environmental factors on neurodevelopment. Many observed effects related to pollutants have been attributed to halogenated aromatic hydrocarbons and to polychlorinated biphenyls (PCBs) in particular [15–17]. Since there is some indication that PCB exposure *in utero* might affect brain sexual differentiation [18–20], a goal of the present work was to quantify the possible effects of a neonatal exposure to Aroclor 1254, a technical mixture containing almost 70 different PCB isomers and congeners [21], on the expression and activity of the aromatase and 5-alpha reductases. In previous studies Aroclor 1254 has been shown to produce neurochemical alterations in several experimental models [22–35] as well as behavioral changes in learning, memory, motor activity, sexual behavior [36–42].

2. Materials and methods

2.1. Animals

Time pregnant Sprague-Dawley (CD SPF/VAF) rats (Charles River, Calco, Italy) were maintained in animal quarters in normal conditions (light schedule: 14 h light–10 h dark). They were fed a standard pellet diet and water was provided *ad libitum*. Sperm-positive vaginal smears were considered as evidence of coitus and this day was designated gestational day (GD) 0. GD16 or GD20 fetuses were delivered by laparatomy and the hypothalamic area of each fetus was used; gender was assessed by the presence of the SRY gene [13]. The Department of Endocrinology Animal Facility fully complies with all Italian current rules concerning the care and use of animals. The number of animals used for each determination (corresponding to the number of samples) is detailed in each figure.

2.2. Neuronal cultures

Neuronal cell cultures were obtained from fetal hypothalami (not sexed) as previously described [43]. Briefly, neuronal cells were obtained from GD16 animals by dispersing the tissue in DMEM. Cells were plated on polylysine-coated 35 mm Petri dishes in phenol red-free DMEM containing 20% fetal calf serum (FCS). After 24 h, the medium was replaced with a chemically defined medium (DMEM without phenol red containing: 50 U/ml penicillin, 50 U/ml streptomycin, 5 μg/ml bovine insulin, 100 μg/ml bovine transferrin, 100 μM putrescine and 20 nM Na selenite). Aromatase activity was assayed after 4 days in culture.

2.3. RNA extraction

The hypothalamic areas of single embryos were removed at gestation day 20 (GD20); total RNA from each tissue fragment was obtained by the acid-phenol method according to Chomczynski and Sacchi [44].

2.4. Aroclor administration in "in vivo"

Aroclor 1254 mixture was kindly provided by Dr. Stephen Safe (Texas AM University). Pregnant rats were given daily Aroclor 1254 (25 mg/kg by gavage) from days 15 to 19 of gestation. The dose was selected on the basis of the studies of Morse et al. [45] demonstrating that this dose schedule exerts specific effects on the rat fetal brain.

2.5. Aroclor administration in "in vitro"

For hypothalamic homogenates (obtained from non-sexed animals), Aroclor 1254 was dissolved in ethanol at a final concentration of 10–100–500 μg/ml and added to the homogenate 1 h before the addition of labeled substrate for the aromatase assay. Control cultures were given ethanol only.

For neuronal cultures, Aroclor 1254 was dissolved in ethanol at a final concentration of 100 μg/ml and added to the neuronal cultures 1 h before the addition of labeled substrate for the aromatase assay. Control cultures were added with ethanol only. In both cases, preincubation with Aroclor 1254 was performed at 37 °C.

2.6. RT-PCR and Southern blot analysis of aromatase, 5-alpha-R1 and 5-alpha-R2

Reverse transcription was performed on 2 μg total RNA from each sample according to manufacturer protocol (GeneAmp RNA PCR Kit, Applied Biosystem, Monza, MI, Italy) using oligo-d(T) (for aromatase and 5-alpha-R2) or random hexamers (for 5-alpha-R1) as primers.

All amplifications were performed on 4 μl of RT mixture (PCR buffer: 50 mM KCl; 10 mM Tris–HCl; 5 mM $MgCl_2$; 1 mM of each dNTP; 1 U/μl RNase inhibitor; 2.5 U/μl MuLV reverse transcriptase) in a final volume of 25 μl using the sense/antisense oligonucleotides (0.5 μM) previously published [7]. The final concentrations of each component of the PCR master mix were maintained as suggested by the manufacturer protocol. Specific annealing temperatures and number of amplification rounds for every PCR were previously established. The amplified products were separated by electrophoresis on 2% agarose gel, identified by ethidium bromide staining and transferred to blotting nylon membrane (Zeta-probe) by capillary elution. Southern blot analysis was performed using the specific [gamma-^{32}P]-labeled oligoprobes (1×10^6 cpm/ml each) [7]. For aromatase and 5-alpha-R2, PCR reactions were performed according to previously published methods (coamplification with neuronal MAP2c) [7]. This normalization was not possible for 5-alpha-R1, since the enzyme is present both in neurons and in glial cells. 5-Alpha-R1 was therefore normalized versus 18S housekeeping gene. Band intensity was quantified by the software Image provided by the National Institute of Health (NIH).

Values are mean ± S.E. of the mean optical density (MOD). The number of samples obtained, each from a single animal, is shown in the figures (numbers inserted at the bottom of the columns).

2.7. Aromatase assay

The aromatase activity in cultured neurons or in hypothalamic homogenates was estimated by the production of ^3H_2O from [1beta-^{3}H]-androstenedione (NEN, Research Product, DuPont, Boston, MA, S.A., 27.5 Ci/mmol), utilizing a procedure previously described [4] with minor modifications. The incubation was conducted with 0.15 μM of [1beta-^{3}H]-androstenedione for 2 h at 37 °C; the reaction media were then transferred in capped tubes containing 40% trichloroacetic acid. The labeled aqueous phases were separated from the unmetabolized substrate and from the steroid products formed during the incubation, by chloroform extraction and 5% charcoal/0.5% dextran precipitation. After a rapid passage through a cotton filter, the tritiated water formed during the arom-