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Sex- and region-specific alterations of progesterone receptor mRNA levels and estrogen sensitivity in rat brain following developmental exposure to the estrogenic UV filter 4-methylbenzylidene camphor

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Abstract

Recently, we reported on in vitro and in vivo estrogenic activity of UV filters and on developmental toxicity of 4-methylbenzylidene (4-MBC) camphor [Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001a. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239; Schlumpf, M., Berger, L., Cotton, B., Conscience-Egli, M., Durrer, S., Fleischmann, I., Haller, V., Maerkel, K., Lichtensteiger, W., 2001b. Estrogen active UV screens. SÖFW–J. 7, 10]. 4-MBC (7, 24, 47 mg/(kg day)) was administered in chow to long Evans rats from 10 weeks before mating of the parent (F0) generation until adulthood of the F1 generation. Peripheral reproductive organs and central nervous system were studied in adult offspring. mRNA expression of progesterone receptor (PR), an estrogen-regulated gene, was investigated in medial preoptic area (MPO) and ventromedial hypothalamic nucleus (VMH) by real-time RT-PCR. We analyzed intact 12-week-old male and female offspring under steady state conditions and adult gonadectomized offspring 6 h after a single s.c. injection of estradiol-17β (E2) (10 or 50 μg/kg) in order to assess estrogen sensitivity. At steady state conditions we observed significantly higher PR mRNA expression in VMH of control females versus control males. 4-MBC exposed females exhibited a decrease in PR mRNA to levels of control males. The increase in PR mRNA in response to E2 was higher in VMH of males of both 4-MBC groups as compared to control males. PR mRNA levels were similar in MPO of control males and females. Developmental 4-MBC exposure increased PR mRNA levels in male MPO, but did not significantly change female levels. The acute response to the lower E2 dose was decreased in MPO of 4-MBC-exposed males, whereas females of the 7 mg/kg dose group exhibited an increased reaction to 50 μg/kg of E2. Our data indicate that developmental exposure to endocrine active chemicals such as the UV filter 4-MBC can interfere with sexually dimorphic gene expression in brain in a sex- and region-specific manner.
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1. Introduction

A number of substances from different groups of chemicals can interact with endocrine systems. These endocrine disrupting chemicals (EDCs) potentially cause adverse effects in humans and wildlife (Damstra et al., 2002). Recently, several ultraviolet (UV) filters frequently used in sunscreens and cosmetics have been found to display estrogen-like activity in vitro and in rats and fish in vivo (Schlumpf et al., 2001a; Tinwell et al., 2002; Holbech et al., 2002; Schreurs et al., 2002; Mueller et al., 2003). Antagonistic action at androgen receptors has also been observed for two UV filters in vitro in MDA-kb2 cells transfected with a luciferase transporter plasmid (Ma et al., 2003). These compounds are released into the environment and because of their lipophilicity have the potential to accumulate in the biosphere, where they have been detected (Nagtegaal et al., 1997). Endocrine active UV filters are addressed in the "CREDO Cluster" of the fifth European Framework Programme (Lorenz, 2003).

Of particular importance are effects of EDCs on endocrine systems during ontogeny, resulting in alterations of organi-

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zational actions of hormones. The action of sex hormones on differentiation processes is not limited to peripheral reproductive organs, but also includes the central nervous system. In mammals, male sexual differentiation of the brain depends on testosterone secretion during a critical time-window, in rats the late fetal and early postnatal periods (MacLusky and Naftolin, 1981). Intraneuronal aromatase of fetal and postnatal brain (George and Ojeda, 1982; Lauber and Lichtensteiger, 1994) converts testosterone to estradiol, which appear to control the great majority of male sexual differentiation processes in central nervous system. Agonistic or antagonistic estrogen receptor ligands may change transcriptional processes in developing brain during critical periods of brain differentiation and cause permanent structural and functional changes.

The estrogenic UV filter 4-methylbenzylidene camphor (4-MBC) has been found to cause a number of developmental and endocrine deficits in rat offspring (Schlumpf et al., 2001b, 2003). The mechanisms underlying these effects are not yet understood. We are testing the hypothesis that at least some of the developmental effects of 4-MBC are caused by persistent changes in the expression of estrogen-regulated genes (estrogen receptors alpha and beta, progesterone receptor, pre-proenkephalin, insulin-like growth factor-I, androgen receptor) in brain and reproductive organs.

In the following, we report on progesterone receptor (PR) mRNA expression in brain. Several studies established the medial preoptic area (MPO) as an important site for the activation of male copulatory behavior in rats (McEwen et al., 1979; Davis and Barfield, 1979; Malsbury, 1971), while the ventromedial hypothalamic nucleus (VMH) plays a prominent role in sexual behavior and receptivity of female rats (Pfaff and Sakuma, 1979). PR in MPO and VMH are regulated by estradiol with region-dependent sex differences (Brown et al., 1987; Lauber et al., 1991). PR mRNA levels were determined by real-time PCR under steady state conditions as well as after acute estradiol injection, in order to assess possible changes in estrogen sensitivity.

2. Material and methods

2.1. Animals and treatment

Long Evans rats were bred in our animal facilities under standard conditions (lights 02.00–16.00 h, $22 \pm 1\,^{\circ}C$) with food and water ad libitum. Males and females of the parent generation (F0) were exposed to 4-MBC in feed for at least 10 weeks before mating. Exposure of dams continued during pregnancy and lactation and in the offspring (F1) until adulthood (12 weeks). 4-MBC was added to chow (Provimi Kliba AG, Kaiseraugst) at three different concentrations (0.1 g/kg, 0.33 g/kg, and 0.66 g/kg chow). Measurements of average diet intake have shown that these dosages correspond to an average daily intake of 7 mg/kg, 24 mg/kg, and 47 mg/kg body weight. Doses were chosen from the acute uterotrophic assay in immature rats (Schlumpf et al., 2001a). The highest chronic dose applied, 70 mg/(kg day), corresponds to 65% of the lowest observed adverse effect level (LOAEL) in the uterotrophic assay, the highest dose used in the present study, 47 mg/(kg day), to 43% of acute LOAEL. The 70 mg/kg dose was well tolerated in adult F0 rats up to 13–14 months, but caused significant early postnatal toxicity (reduced survival rate), and therefore, was not used for gene expression studies. The dose range has meanwhile been extended to include 0.7 mg/(kg day), but data on central nervous system are not yet available for that dose.

2.1.1. Experiment 1: Steady state

Part of the offspring were sacrificed without further experimental manipulation at 12 weeks of age, between 14.00 and 16.00, females always at the stage of metestrous (= diestrus 1) controlled by vaginal smear.

2.1.2. Experiment 2: Acute estrogen challenge

Female and male offspring were gonadectomized at the age of 10 weeks (anesthetisia: fentanyl, fluanison; medetomidine, atropine). Two weeks later, the animals were given a single subcutaneous injection of estradiol-17β (E2) (Calbiochem Lucerne, Switzerland), 10 or 50 μg/kg, dissolved in DMSO, or of the vehicle (DMSO, Fluka, Switzerland). Six hours after the injection, the animals were killed by decapitation.

2.2. Tissue preparation

The brains were removed, quickly frozen in 2-methylbutane cooled by liquid nitrogen, and stored at $-80\,^{\circ}C$ until use. For dissection of the two sexually dimorphic brain regions MPO and VHM, the brain was placed into a brain matrix (Bilany RBM3000C) and cut into 1 mm coronal slices with razor blades. The two regions were punched from the frozen tissue with a stainless-steel needle (VMH, Ø 0.9 mm; MPO, Ø 1.9).

2.3. Determination of mRNA

2.3.1. RNA isolation and reverse transcription

Tissue pieces were disrupted in 350 μl RLT buffer of the RNeasy-mini kit (Qiagen) and homogenized by a sonicator. Extraction of total tissue RNA was performed with RNeasy-mini kit according to manufacturer's instructions. Genomic DNA was thoroughly digested by DNase-I. To check for DNA contamination total RNA was tested on an ethidium bromide-stained 2.5% agarose gel. cDNA was obtained from total RNA by using the TaqMan® Reverse Transcription Reagents kit according to the manufacturer's instructions (Applied Biosystems, Rotkreuz, Switzerland).

2.3.2. Real-time RT-PCR

mRNA levels of PR were quantitated by real-time PCR using the ABI PRISM 7700 Sequence Detection System (PE

Applied Biosystems, Rotkreuz, Switzerland) and TaqMan universal PCR master mix (Applied Biosystems). mRNA sequences for PR were derived from NCBI (National Center for Biotechnology Information) gene bank. Primers and TaqMan probe were designed with PrimerExpress Software, Version 2.0 (Applied Biosystems), and ordered from Microsynth (Balgach, Switzerland). The amount of PR mRNA was calculated using the relative standard curve method with cyclophilin A mRNA as reference gene.

2.4. Statistical analysis

Treatment groups were compared by two-way analysis of variance followed by pairwise comparisons with Bonferroni correction; $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Steady state levels of PR mRNA in VMH and MPO

3.1.1. VMH

Progesterone receptor mRNA levels in VMH exhibited a significant sex difference in adult control offspring with higher PR mRNA in females than in males (Fig. 1). Females of the 4-MBC-exposed groups showed a decrease of PR mRNA that was statistically significant at the lowest 4-MBC dose (7 mg/(kg day)), the higher two doses exhibited the same tendency. PR mRNA in VMH of male offspring was not significantly affected by 4-MBC treatment.

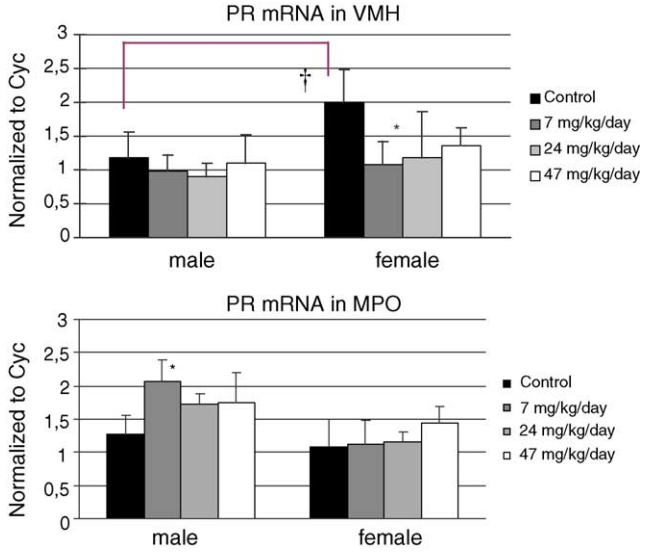

Fig. 1. Progesterone receptor (PR) mRNA levels in ventromedial hypothalamic nucleus (VMH) and medial preoptic area (MPO) of adult rat offspring exposed to 4-methylbenzylidene camphor (4-MBC, 7, 24, or 47 mg/(kg day)) in chow during pre- and postnatal life. Mean ± S.D., VMH, $n = 7–8$; MPO $n = 6$. PR mRNA level normalized to cyclophilin. Difference from untreated control $^{*}p < 0.05$, difference between control males and females $^{\dagger}p < 0.01$.

3.1.2. MPO

In contrast to VMH, PR mRNA levels of MPO were similar in adult control males and females (Fig. 1). Developmental exposure of males to 4-MBC resulted in an increase of PR mRNA levels that was statistically significance at the lowest dose of 4-MBC (7 mg/(kg day)). No significant treatment-induced changes were seen in female MPO.

3.2. Effect of acute estrogen challenge on PR mRNA

3.2.1. VMH

In VMH of untreated controls, induction of PR mRNA by a single s.c. injection of estradiol differed significantly between sexes. The lower dose of estradiol (10 μg/kg) induced PR mRNA only in females, whereas the higher dose (50 μg/kg) elicited a response of similar magnitude in both sexes (Fig. 2). 4-MBC exposed males of both 4-MBC dosage groups exhibited increased induction of PR mRNA compared to untreated controls. The magnitude of the response reached female levels (Fig. 2, bottom). In contrast, an effect of 4-MBC exposure on the magnitude of PR mRNA induction in female VMH was seen only in the 7 mg/(kg day) group after 50 μg/kg estradiol.

3.2.2. MPO

MPO exhibited a different effect pattern (Fig. 3). In contrast to VMH, the magnitude of PR mRNA induction by estradiol was similar in male and female untreated controls also after the lower dose of estradiol (Fig. 3, bottom). Enhanced induction of PR mRNA was present only in females exposed to 4-MBC at 7 mg/(kg day) after 50 μg/kg estradiol. 4-MBC exposed males exhibited a significant, dose-dependent reduction, rather than increase, of the effect of estradiol on PR mRNA at the low dose of estradiol (10 μg/kg), while the response to 50 μg/kg estradiol did not differ from controls.

Thus, developmental exposure to 4-MBC can interfere with the regulation of an estrogen target gene such as progesterone receptor. The chemical abolishes the sexual dimorphism of PR mRNA steady state levels in VMH reducing female levels to the lower male level. PR mRNA in MPO is also affected, but with a different effect pattern. In line with earlier reports (Brown et al., 1987; Lauber et al., 1991), regulation of PR expression in untreated controls was found to be region- and sex-specific.

Experiments designed to assess the efficiency of estradiol to induce PR mRNA expression may provide more direct information on possible changes in gene regulation than data on changes in steady state mRNA levels, which may result from interactions with various processes. The effect of developmental 4-MBC exposures on PR mRNA induction by E2 in gonadectomized rats was again sex- and region-specific. In males, the increased responsiveness in VMH contrasted with a decreased response in MPO, while females of the low 4-MBC dose group exhibited increased sensitivity to the higher estradiol dose in both brain regions. The combination of changes in steady state levels and sensitivity to

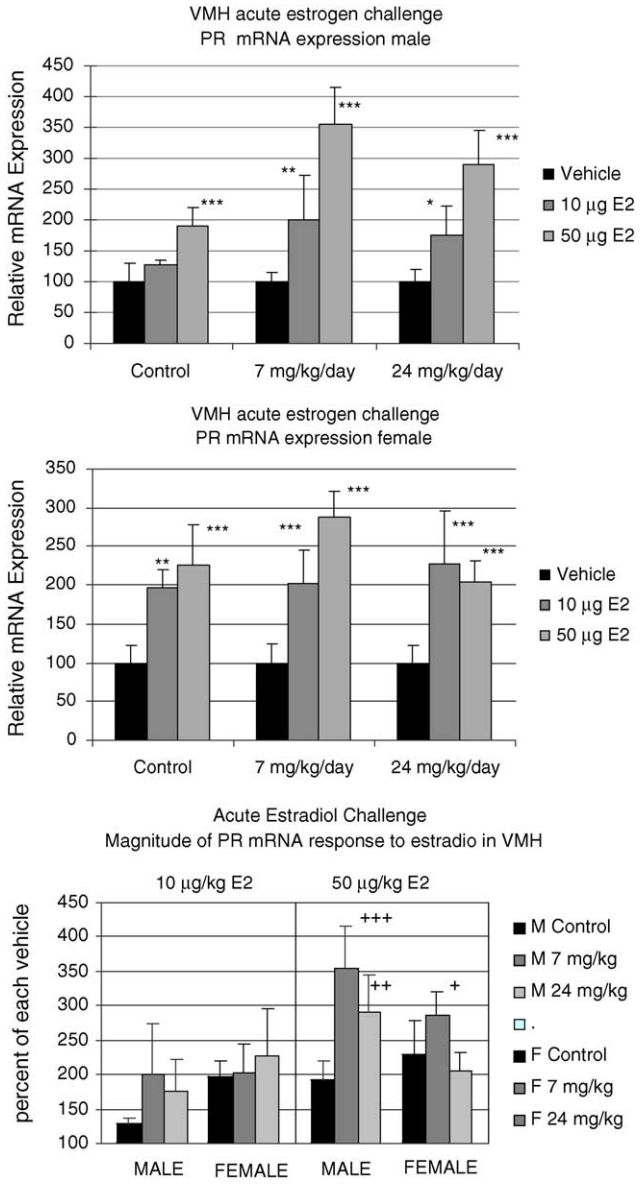

Fig. 2. Effect of a single estradiol-17β (E2) injection (10 or 50 μg/kg s.c.) on progesterone receptor (PR) mRNA levels in ventromedial hypothalamic nucleus (VMH) of adult gonadectomized male and female rat offspring from untreated control and 4-MBC-exposed groups. Top and center: PR mRNA levels in VMH of E2-injected males and females relative to PR mRNA of vehicle-injected group (mean ± S.D., $n = 6$). Difference from vehicles $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Bottom: change induced by E2 in untreated (M and F Control) or 4-MBC-exposed (M, F, 7, or 24 mg/(kg day)) gonadectomized male and female offspring expressed as percentage of the PR mRNA level of the vehicle-injected groups of each of control or treatment groups. Difference from level of untreated control groups $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$. Increases induced by 10 μg/kg E2 in untreated controls differ between males and females for $p < 0.05$.

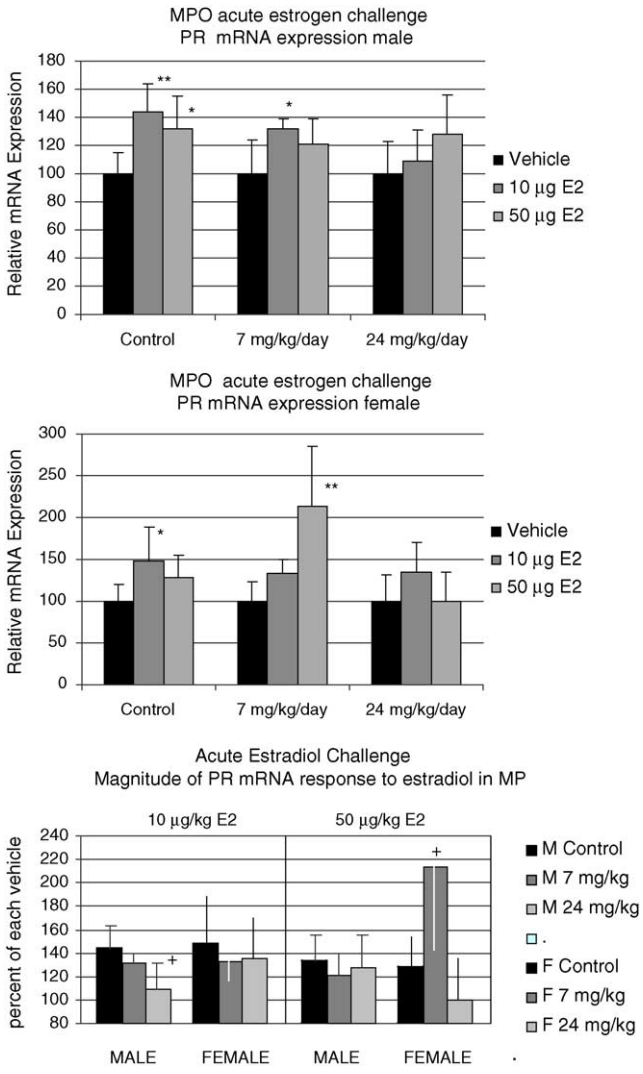

Fig. 3. Effect of a single estradiol-17β (E2) injection (10 or 50 μg/kg s.c.) on progesterone receptor (PR) mRNA levels in medial preoptic area (MPO) of adult gonadectomized male and female rat offspring from untreated control and 4-MBC-exposed groups. Top and center: PR mRNA levels in MPO of E2-injected males and females relative to PR mRNA of vehicle-injected group (mean ± S.D., $n = 6$). Difference from vehicle $^{*}p < 0.05$, $^{**}p < 0.01$. Bottom: Change induced by E2 in untreated (M and F control) or 4-MBC-exposed (M, F, 7 or 24 mg/(kg day)) gonadectomized male and female offspring expressed as percentage of the PR mRNA level of the vehicle-injected groups of each of control or treatment groups. Difference from level of untreated control group $^{+}p < 0.05$.

estrogen creates a complex situation that is difficult to interpret in functional terms. However, several lines of evidence, including data from intrahypothalamic PR mRNA antisense injection (Ogawa et al., 1994), indicate that the induction of PR mRNA by estrogen in VMH is intimately linked with the induction of female sexual behavior.

Our data indicate that the estrogenic UV filter 4-MBC which is frequently being used in sunscreens and cosmetics has the potential to interfere with the expression of estrogen-regulated genes in brain consequent to developmental exposure. This may lead to alterations in brain function, in particular in neuroendocrine circuits. Exposure levels in humans have not been published so far. The systemic exposure dose for humans has been estimated to be 0.23 mg 4-MBC/kg body weight (SCCNFP, 1998). The present lowest observed adverse effect level for CNS effects in rats is only 30 times higher. However, we think that indirect calculations of this

kind provide an insufficient basis for risk assessment. We are presently conducting chemical–analytical studies in rat fat tissue and plan to correlate these data with concentration data from human milk, in order to obtain a precise concentration ratio.

On a more general level, the example of developmental 4-MBC exposure demonstrates that sex hormone-regulated genes in CNS are a potentially sensitive target of endocrine active chemicals.

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