Effects of Perinatal Exposure to Phthalate/Adipate Esters on Sex Steroid Levels and Hypothalamic Gene Expression during Early Postnatal Periods in Rats

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ABSTRACT

Our previous research has identified granulin (grn) and p130 genes as sex steroid-inducible genes in the rat hypothalamus, which might be involved in sexual differentiation of the brain. Phthalate esters that are used as plasticizers and also found at low levels in foods such as dairy products are often mentioned as suspected endocrine disrupters. The purpose of the present study is to elucidate whether perinatal exposure to di-n-butyl phthalate (DBP), diisononyl phthalate (DINP) and di-2-ethylhexyl adipate (DEHA) affects hypothalamic sex steroid-inducible genes. The present study assessed the effects of perinatal exposure to DBP, DINP and DEHA on sex steroid hormones levels and hypothalamic grn and p130 mRNA expressions at postnatal day (PND) 3 and 7. Pregnant rats were fed a soy-free diet containing 20, 200, 2,000 and 10,000 ppm of DBP, 40, 400, 4,000 and 20,000 ppm of DINP, or 480, 2,400 and 12,000 ppm of DEHA from gestational day (GD) 15 to GD 3 or 7. At PND 3 and 7, perinatal exposure to these chemicals did not substantially affect serum concentrations of testosterone and estradiol. At PND 3, the expression of grn mRNA levels in males was decreased by DEHA, and that of p130 was decreased by DBP, DINP and DEHA, though the effects were not dose-dependent. At PND 7, the expression of grn gene in female pups was increased by higher doses of DBP and all the doses, except for 4,000 ppm, of DINP, while that in male pups decreased by 480 and 12,000 ppm of DEHA. Hypothalamic expression of p130 mRNA in males was increased by lower doses of DBP and all the doses of DINP, whereas that of females was decreased by 480 and 2,400 ppm of DEHA. These results suggest that these chemicals may affect the expression of grn and p130 genes by directly acting on the hypothalamus, thus leading to inappropriate expression of these genes.

(Key words : Adipate, Granulin, Hypothalamus, p130, Phthalate)

INTRODUCTION

In the mammals, it is well established that estradiol, the product of aromatizable testosterone, mediates most aspects of sexual differentiation during a restricted developmentally sensitive period, known as the critical period. In this period, testosterone secreted by the testes in male fetuses and neonates acts to masculinize and deffeminize areas of the developing brain after the brain cellular metabolism of testosterone to estradiol by the enzyme aromatase (McDonald *et al.*, 1970; MacLusky and Naftolin, 1981). In rats, this critical period is operationally defined by the onset of testicular secretion on embryonic day 18 and is terminated when females become refractory to the masculinizing effects of exogenous testosterone (or estradiol). The termination of the critical period is variable for different functional endpoints and ranges from postnatal day (PND) 6 to PND 10 (Diaz *et al.*, 1995).

Generally the steroid hormones such as androgen and estrogen bind to respective intracellular receptors, and the steroid-receptor complexes transfer into nucleus. They bind to the variety of transcription factors and activate transcription of the various genes. The expression of the novel genes and proteins were reported in the brain by neonatal treatment with androgen and estrogen (Stanley *et al.*, 1986; Stanley and Fink, 1986), and neonatal treatment with inhibitor of RNA polymerase, that has a critical role in the gene expression, inhibits the brain sexual differentiation (Salaman and Birkett, 1974). These results suggest that the role of steroids in the brain sexual differentiation also involves the gene transcription. Thus, it is an important step for investigating the mechanism

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of the brain sexual differentiation to isolate the genes and their products induced by the sex steroids in the critical period of the brain sexual differentiation. We have previously identified the granulin (grn) precursor gene (Suzuki *et al.*, 1998) and p130 gene (Yonehara *et al.*, 2002) as an androgen-inducible gene in the rat hypothalamus during the critical period and demonstrated that these genes are involved in sexual differentiation of the rat brain (Suzuki *et al.*, 2000, 2001; Suzuki and Nishiahara, 2002; Yonehara *et al.*, 2002). According to our observations of the androgen- and estrogen-dependent induction of grn and p130 gene and of the sexually different patterns of their gene expression in the hypothalamus, it is possible that these genes are good parameter for assessing the sex steroid action of environmental endocrine disruptors on the neonatal brain (Suzuki and Nishiahara, 2002).

Endocrine disrupting chemicals (EDCs) include a number of environmental chemicals and chemicals found in plant products that interact with an endocrine system, often due to their activity as a hormonal mimic (Waldron and Naber, 1974). The greater part of EDCs are recognized to bind estrogen receptors, which are members of a family of ligand-activated proteins that serve as transcriptional factors for a wide variety of cellular target genes, and act as agonists or antagonists of estrogen (Colborn *et al.*, 1993; Barlow *et al.*, 1999). During recent years the phthalates have attracted much attention because several of these compounds are suspected of possessing endocrine disrupting effects. Phthalate esters are used as plasticizers in certain infant toys and consumer products (e.g., food wraps, containers for soaps, shampoos, and perfumes) and medical devices such as tubings and catheters. They have been also found at low levels in foods, particularly fatty foods such as dairy products because they are fat-soluble (Sharman *et al.*, 1994; Page and Lacroix, 1995). Recently, considerable concern has focused on the health effects of phthalate exposures to children from toys and other sources, and to pregnant woman from dialysis treatment or blood transfusions.

In utero exposure to DBP (Gray *et al.*, 1999; Mylchreest *et al.*, 1999), DINP (Gray *et al.*, 2000) and di (2-ethylhexyl) phthalate (DEHP) (Arcadi *et al.*, 1998; Gray *et al.*, 1999) have been shown to disrupt differentiation of androgen-dependent tissues in male rat offspring. Mainly, because some phthalates induce testis toxicity and antiandrogenic effects, alternative compounds, di (2-ethylhexyl) adipate (DEHA), have replaced the phthalate as plasticizers in flexible polyvinyl chloride (PVC) products (Petersen and Breindahl, 1998) like medical devices, plastic wrapping, and toys. However, although DEHA is currently being evaluated as potential substitutions for some phthalates, due to similarities in structure and metabolism of DEHP and DEHA, and of DEHA and DINP, it may be hypothesized that similarities in action may also exist.

In the present study, we assessed the effects of perinatal exposure to DBP, DINP and DEHA on sex steroids and hypothalamic gene expression in rats. Few data on the change of these chemicals-mediated hypothalamic gene expressions have been published. Moreover, there have been no reports addressing the effect of these chemicals on the brain sexual differentiation in rats. To this end, serum sex steroid levels and hypothalamic gene expression of grn and p130 at PND 3 and 7 were examined.

MATERIAL AND METHODS

Test Compounds

DBP (CAS No. 84-74-2, purity >98%, Cat No. P0292) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). DINP (CAS No. 28553-12-0, purity >98%, Cat No. 040-22805), and DEHA (CAS No. 103-23-1, purity >99%, Cat No. 027-13006) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animals and Treatments

This study followed Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. Wistar-Imamichi rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) of approximately 8 weeks of age were obtained and maintained in a room with controlled illumination (lights on 0500~1900 o'clock) and temperature (23±1℃), and provided chow and water *ad libitum*. Animals were mated (mating confirmed by sperm presence in vaginal smears), and the day of mating was designated as gestational day (GD) 0. The pregnant females were allowed to deliver pups naturally (day of birth = PND 0), and the litter size was adjusted to eight on the PND 5. Some of the pups born from the control diet-dams were subcutaneously injected with 20 μg estradiol benzoate (EB; Sigma) or 10 mg testosterone propionate (TP; Sigma) in 50 μl sesame oil on PND 2. The dams were fed with pulverized soy-free diet (NIH-07-PLD (phytoestrogen-low diet); Oriental yeast Co. Ltd., Tokyo, Japan) to reduce the effect of phytoestrogen. From GD 15 to the PND 7, the dams were provided with pulverized soy-free diet that contained 20, 200, 2,000 and 10,000 ppm of DBP, 40, 400, 4,000 and 20,000 ppm of DINP, or 480, 2,400 and 12,000 ppm of DEHA.

Preparation of Hypothalamus and Serum

At PND 3 and 7, pups were sacrificed by decapitation. The brain was immediately removed and the entire hypothalamus, bordered anteriorly by the optic chiasma, laterally by the hypothalamic fissures, and posteriorly by

the mammillary body, was dissected out (Suzuki *et al.*, 2001). Hypothalamic blocks were frozen in liquid nitrogen, and stored at - 80℃ until RNA isolation. Trunk blood was taken and centrifuged at 3,000 rpm for 15 min at 4℃. The serum was collected and stored at - 20℃ until assayed for serum testosterone and estradiol concentrations.

Real-time Quantitative RT-PCR

Total RNA was isolated from hypothalamus using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed into cDNA using GeneAmp RNA PCR Kit (Applied Biosystems, NJ, USA) in a reaction volume of 20 μl according to the manufacturer's protocol. RT-PCR was performed on LightCycler (Roche Diagonostics GmbH, Mannheim, Germany) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagonostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The primer sets for rat grn (forward primer; 5'-AGTTCGAATGTCCTGACTCCGCCA-3', reverse primer; 5'-AAGCCACTGCCCTGTTGGTCCTTT-3'), rat p130 (forward primer; 5'-GTGTAAGTGCTGCCTCGGGCACAG - 3', reverse primer; 5'-CGGGAACATGACCTGAGTAT-CTGTTC-3') and rat 40S ribosomal protein S29 (RPS29) (forward primer; 5'-TGAAGGCAAGATGGGTCACCAG-CAGC-3', reverse primer; 5'-CAGGGTAGACAGTTGGT-TTCATTGGG-3') were used. Rat-specific primers were designed using Primer3 software (available at http://fokker.wi.mit.edu/primer3/) with the following parameters: minimum Tm = 68℃, maximum Tm = 73℃, optimum Tm = 70℃, product size range = 180 to 250 base pairs (bp), and primer length = 23 to 25 bp, optimum length = 24 bp. To construct a relative standard curve for each gene, PCR amplification was performed with template dilutions ranging from 10^1 to 10^3 copies. PCR amplification was performed under the following conditions: each cycle involved 15 sec at 95℃ for denaturing, 15 sec at 60℃ for annealing, and 15 sec at 72℃ for extension with 40 cycles. The cDNA was diluted for hundred-fold prior to PCR amplification.

Hormone Assays

Serum concentrations of testosterone and estradiol were measured by testosterone and estradiol enzyme-immunoassays (EIA) kits (Cayman Chemical, MI, USA), respectively, according to the manufacturer's protocol.

Statistical Analysis

Statistical analyses were conducted using StatView (version J5, Abacus Concepts, Inc.). A one-way analysis of variance and Dunnett's test were used to determine differences between treated and control groups. Differences were considered statistically significant at $p<0.05$.

RESULTS

Sex Steroid Levels

In the control group at PND 3, serum testosterone levels were significantly higher in males than females (Fig. 1A), while serum estradiol levels were not different between sexes (Fig. 1B). EB or TP-treatment significantly increased serum estradiol and testosterone levels, respectively. These patterns at PND 7 were similar to that of PND 3. At PND 3, the perinatal exposure to DBP, DINP and DEHA did not affect concentration of serum testosterone and estradiol as compared with the control group in both sexes, with exception that 40 and 20,000 ppm DINP-exposed males and 200 and 2,000 ppm DBP-exposed females were significantly increased testosterone and estradiol levels, respectively. At PND 7, serum concentration of testosterone and estradiol was not changed by perinatal exposure to these chemicals, however, DINP of 40 ppm significantly decreased estradiol levels in female pups (Fig. 2).

Hypothalamic Gene Expression

At PND 3, hypothalamic expression of grn and p130 mRNA was higher in males than females (Fig. 3), while that of grn and p130 were far more expressed in males and females than opposite sexes on PND 7, respectively (Fig. 4). EB or TP treatment in female was used as positive control of theses genes expression, because these

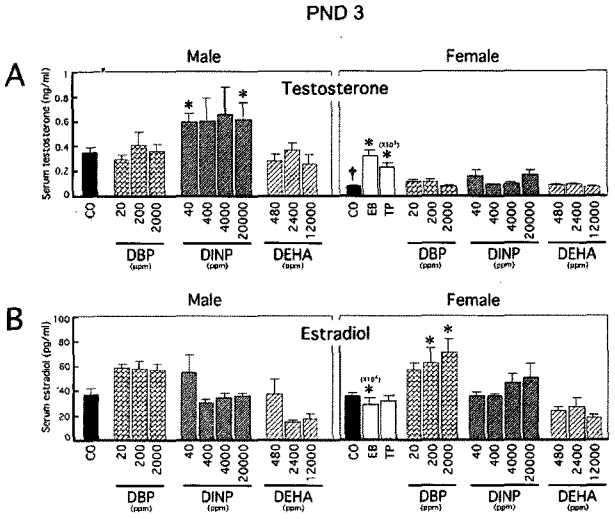

Fig. 1. Serum testosterone (A) and estradiol (B) levels at postnatal day 3. The rats exposed to DBP, DINP and DEHA from gestational day 15 to postnatal day 3. Data represent mean ± SE, n=5~7 pups from five to six litters per group. The column and vertical bar is multiplied by the value in parentheses. CO, control diet; EB, estradiol benzoate; TP, testosterone propionate; DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DEHA, di-2-ethylhexyl phthalate. * $p<0.05$ vs. CO; † $p<0.05$ males vs. females.

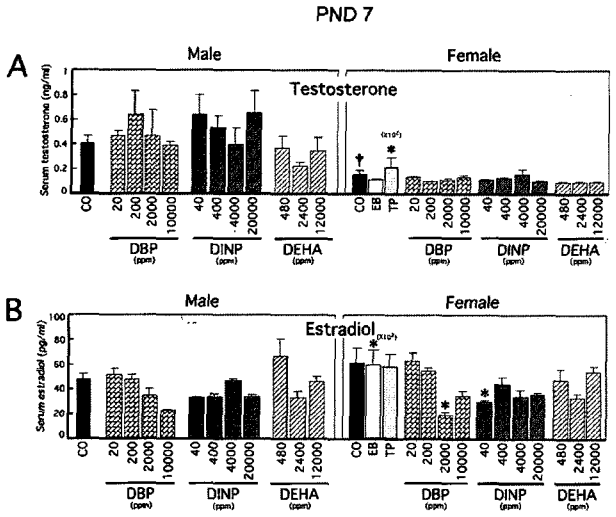

Fig. 2. Serum testosterone (A) and estradiol (B) levels at postnatal day 7. The rats exposed to DBP, DINP and DEHA from gestational day 15 to postnatal day 7. Data represent mean ± SE, n=5~7 pups from five to six litters per group. The column and vertical bar is multiplied by the value in parentheses. CO, control diet; EB, estradiol benzoate; TP, testosterone propionate; DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DEHA, di-2-ethylhexyl phthalate. * $p< 0.05$ vs. CO; † $p< 0.05$ males vs. females.

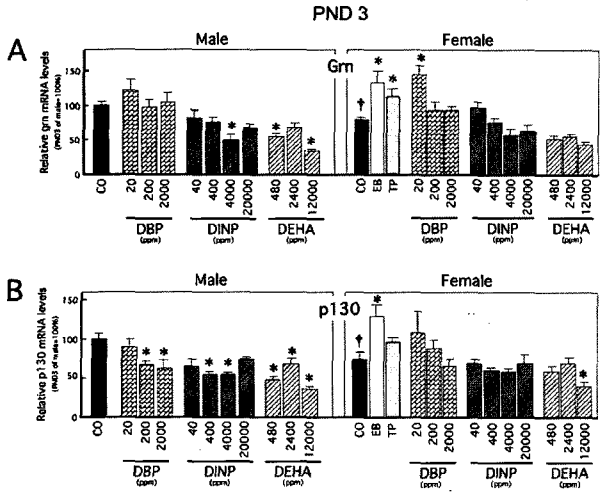

Fig. 3. Expression of grn (A) and p130 (B) mRNA in neonatal hypothalamus at postnatal day 3. The rats exposed to DBP, DINP and DEHA from gestational day 15 to postnatal day 3. Each value is normalized using RPS 29. Data represent mean ± SE, n=5~7 pups from five to six litters per group. CO, control diet; RPS 29, rat 40S ribosomal protein S 29; EB, estradiol benzoate; TP, testosterone propionate; DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DEHA, di-2-ethylhexyl phthalate. * $p< 0.05$ vs. CO; † $p< 0.05$ males vs. females.

genes expression are enhanced by this treatment. At PND 3, the expression of grn gene in male pups did not change by exposure to these chemical, except for 4,000 ppm

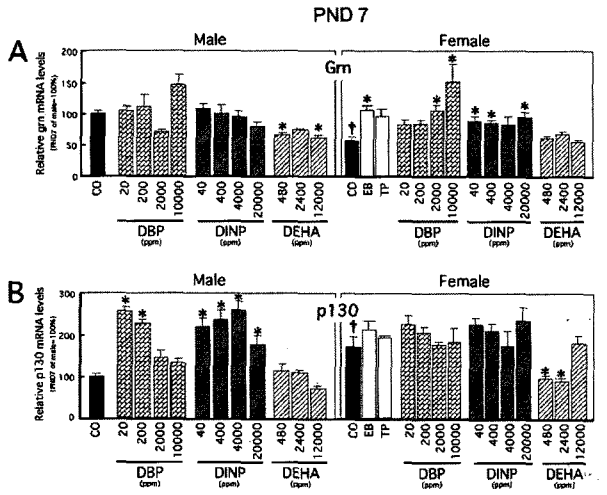

Fig. 4. Expression of grn (A) and p130 (B) mRNA in neonatal hypothalamus at postnatal day 7. The rats exposed to DBP, DINP and DEHA from gestational day 15 to postnatal day 7. Each value is normalized using RPS 29. Data represent mean ± SE., n=5~7 pups from five to six litters per group. CO, control diet; RPS 29, rat 40S ribosomal protein S 29; EB, estradiol benzoate; TP, testosterone propionate; DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DEHA, di-2-ethylhexyl phthalate. * $p< 0.05$ vs. CO; † $p< 0.05$ males vs. females.

of DINP and 480 and 12,000 ppm of DEHA (significant decreased in these doses), while that in 20 ppm DBP exposed female pups was significantly increased (Fig. 3A). On the other hand, p130 gene expression in female pups was significantly decreased only by 12,000 ppm of DEHA, whereas that in male pups was decreased by DBP (200 and 2,000 ppm), DINP (400 and 4,000 ppm) and all the doses of DEHA (Fig. 3B).

At PND 7, hypothalamic expression of grn mRNA was higher in males (Fig. 4A), and that of p130 mRNA was higher in females (Fig. 4B). The expression of grn gene in female pups was increased by higher doses (2,000 and 10,000 ppm) of DBP and all the doses, except for 4,000 ppm, of DINP, while that in male pups decreased by 480 and 12,000 ppm of DEHA (Fig. 4A). Hypothalamic expression of p130 mRNA in males was increased by lower doses (20 and 200 ppm) of DBP and all the doses of DINP, whereas that of females was decreased by 480 and 2,400 ppm of DEHA (Fig. 4B).

DISCUSSION

Although reductions in fetal testosterone levels following gestational exposure to phthalate have been widely observed in male rats, in the present study, perinatal exposure to DBP, DINP and DEHA did not substantially affect serum sex steroid levels at PND 3 and 7. However,

a decrease in anogenital distances (AGD) (data not shown) probably by reducing fetal testicular testosterone production was observed at PND 1. Therefore, these results imply that fetal testosterone levels were lower than that of control group by exposure to DINP and DEHA during gestational period, resulting in decreased AGD.

On the other hand, it is not clear why sex steroid levels were high in 40 and 20,000 ppm of DINP-exposed males and 200 and 2,000 ppm of DBP-exposed females at PND 3. Although these reasons are unknown in the present study, at least, there exists one possibility; sex steroid-binding protein (SBP) (produced by the liver), also known as sex hormone-binding globulin (SHBG), was related with this phenomenon. In humans and most vertebrate species, the sex steroids circulate in the bloodstream bound to SBP and are thus not freely available to target cells. It is generally believed that the SBP protects steroids from rapid metabolic degradation and plays a role in regulating the amount of steroid that is available to target tissues (Rogers and Kavlock RJ, 1998). Thus, the hormone-SBP complex may remain longer than hormones without SBP. DBP and DINP may modulate SBP levels through interaction with liver. Increased sex steroid levels may result from the increased SBP levels by DBP and DINP. These facts are supported by Tollefsen *et al.* (2002) who reported that DBP and potent estrogen mimics are able to induce a substantial up-regulation of circulating levels of SBP *in vivo*.

In the present study, gene expression of grn in the hypothalamus was higher in males at PND 3 and 7, and that of p130 was higher in females at PND 7, which were consistent with our previous studies (Suzuki *et al.*, 1998; Yonehara *et al.*, 2002) (Fig. 5). Both DBP and DINP increased grn mRNA levels in female neonates at PND 7, though not dose-dependently, without affecting those in males. The phthalates may affect the expression of these genes by directly acting on the hypothalamus, because they did not substantially affect serum sex steroid levels at PND 3 and 7.

Several *in vitro* studies have shown that phthalates such as DBP and butylbenzyl phthalate (BBeP) are capable of binding to estrogen receptor-(ER), inducing ER-mediated gene expression, and enhancing the proliferation of MCF-7 human breast cancer cells expressing ER (Jobling *et al.*, 1995; Harris *et al.*, 1997; Zacharewski *et al.*, 1998; Andersen *et al.*, 1999). Since Suzuki *et al.* (2001) have previously shown that androgen upregulates grn gene expression after conversion to estrogen, the increase in grn gene expression in female hypothalamus may be due to the estrogenic properties of the pthalates. The reason why the effects of the chemicals were not dose-dependent is currently unclear, but similar non-dose-dependent effect of DBP has been reported by others (Masutomi *et al.*, 2003). On the contrary, DBP and DINP increased p130 mRNA levels in only male neonates at PND 7, though again not dose-dependently. The

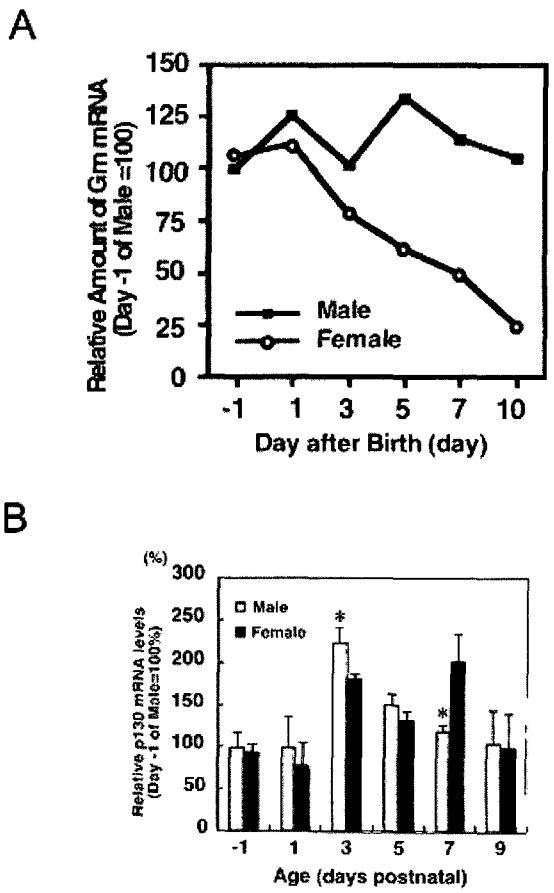

Fig. 5. Changes in grn (A; Suzuki et al., 1998) and p130 (B; Yonehara et al., 2002) mRNA expression levels in the hypothalamus of intact male and female rats during the perinatal period. Each value was normalized using the G3PDH (A) and RPS 29 (B) value, relative to the defining value of in males 1 day before birth (−1).

anti-andorogenic properties of these compounds, especially in the case of DINP, may account for this increase in p130 gene expression. The effect of DEHA on gene expression was also sex specific, i.e., it decreased grn and p130 mRNA levels in males and females, respectively. Contrary to the pthalates, the adipate might exert antiandrogenic effects on males and androgenic effects on females, but further studies are needed to clarify the presice mechanisms underlying the actions of pthalate/adipate esters on sex steroid-dependent and time course gene expression in the hypothalamus. The results obtained from present study are summarized in Table 1.

In conclusion, sex steroid levels were not largely changed by the exposure to DBP, DINP and DEHA, while hypothalamic gene expression pattern at PND 3 and 7 showed considerable changes. These results suggest that these chemicals may affect the expression of grn and p130 genes by directly acting on the hypothalamus, thus leading to inappropriate expression of these genes.

Table 1. Summary of the present study

Day	Parameter	DBP	DINP	DEHA
PND 3	T	M; NE F; NE	M; ↑ (40, 20000) F; NE	M; NE F; NE
	E2	M; NE F; ↑ (200, 2000)	M; NE F; NE	M; NE F; NE
PND 7	T	M; NE F; NE	M; NE F; NE	M; NE F; NE
	E2	M; NE F; ↓ (2000)	M; NE F; ↓ (40)	M; NE F; NE
PND 3	Grn	M; NE F; ↑ (20)	M; ↓ (4000) F; NE	M; ↓ (480, 1200) F; NE
	p130	M; ↓ (200, 2000) F; NE	M; ↓ (400, 4000) F; NE	M; ↓ (A) F; ↓ (12000)
PND 7	Grn	M; NE F; ↑ (2000, 10000)	M; NE F; ↑ (40, 400, 20000)	M; ↓ (480, 12000) F; NE
	p130	M; ↑ (20, 200) F; NE	M; ↑ (A) F; NE	M; NE F; ↓ (480, 12000)

Values in parentheses are dose levels (ppm). M, male; F, female; NE, no effects; A, at all the doses; DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DEHA, di-2-ethylhexyl phthalate; ↑, Statistically significant increase; ↓, Statistically significant decrease.

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