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Specific effects of prenatal DEHP exposure on neuroendocrine gene expression in the developing hypothalamus of male rats

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Abstract Endocrine disrupting chemicals may disrupt developing neuroendocrine systems, especially when the exposure occurs during a critical period. This study aimed to investigate whether prenatal exposure to di-(2-ethylhexyl) phthalate (DEHP), a major component of plasticizers used worldwide, disrupted the development of a network of genes important for neuroendocrine function in male rats. Pregnant rats were treated with corn oil (vehicle control), 2, 10 or 50 mg/kg DEHP by gavage from gestational day 14 to 19. The neuroendocrine gene expressions were quantified using a 48-gene Taqman qPCR array in the whole hypothalamus of neonatal rats (postnatal day 1) and in the anteroventral periventricular nucleus (AVPV), medial preoptic nucleus (MPN) and arcuate nucleus (ARC) of adult rats (postnatal day 70). Immunofluorescent signals of ERα and CYP19 were detected using the confocal microscopy in adult AVPV, MPN and ARC. The results showed that prenatal DEHP exposure perturbed somatic and reproductive development of offspring. Eleven genes were down-regulated in neonatal hypothalamus and showed non-monotonic dose–response relationships, that the 10 mg/kg DEHP dosage was associated with the greatest number of gene expression changes. Different from this, 14 genes were altered in adult AVPV, MPN and ARC and most of alterations were found in the 50 mg/kg DEHP group. Also, 50 mg/kg DEHP reduced ERα expression in the ARC, but no alterations were observed in CYP19 expression. These results indicated that prenatal DEHP exposure may perturb hypothalamic gene programming and the influences are permanent. The effects showed dependence on developmental stages and nuclei region.

Keywords Endocrine-disrupting chemicals (EDCs) · Di-(2-ethylhexyl) phthalate (DEHP) · Hypothalamus · Neuroendocrine gene · Developmental disorder

Introduction

Phthalate esters are a group of industrial chemicals that are primarily used as plasticizers of polyvinyl chloride. Among the varieties of phthalate esters, di-(2-ethylhexyl) phthalate (DEHP) represents about 50% of plasticizers used worldwide (ECPI 2010). DEHP can be found in building materials, flooring, toys, food packaging, personal care products and medical devices, and DEHP exposure is continuous and widespread for the general population (Guo and Kannan 2011; Schecter et al. 2013; Silva et al. 2004).

As a well-known endocrine-disrupting chemical (EDC) with anti-androgenic effects, DEHP and its metabolites are associated not only with reproductive dysfunction in adult male mammals, but also with the development of male offspring and the subsequent manifestation of long-term effects in rats and humans (Foster 2006; Specht et al. 2014; Swan 2008). In animal studies, DEHP exposure in utero shortened anogenital distance (AGD), induced undescended testicles,

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reduced testis and seminal vesicle weight, and decreased serum testosterone (*T*) concentrations and semen quality (Christiansen et al. 2010; Gray et al. 2000; Pocar et al. 2012). In humans, maternal DEHP metabolite levels in urine were associated with a decreased ratio of *T* to estradiol (*E*2) in cord blood of male neonates, shortened AGD in male infants, and decreased semen volume in adults (Araki et al. 2014; Axelsson et al. 2015; Suzuki et al. 2012; Swan et al. 2005).

Although much is known about phthalate actions on the male reproductive tract, the control of reproductive function is dependent upon normal development of the hypothalamus and pituitary cells that, together with the gonad, constitutes the reproductive axis (Gore et al. 2011; Lomniczi et al. 2013; Mayer et al. 2009). Increasing evidence indicates that central neuroendocrine systems are the targets of EDC and may be perturbed by EDC exposures during critical development stages in utero (Gore 2001; Gore et al. 2011). There is also limited evidence on a handful of genes showing that prenatal phthalate exposures affect the hypothalamus. Lee et al. reported that dibutyl phthalate (DBP) and diisononyl phthalate (DINP) exposure during prenatal period increased granulin and *p130* mRNA levels in the hypothalamus of male rat offspring (Lee et al. 2006). In a study by Takagi et al., the expression of progesterone receptor in the hypothalamus of female offspring rats was down-regulated by perinatal exposure to 20,000 ppm DINP (Takagi et al. 2005). When Carbone et al. treated rat dams with DEHP from pregnancy onset to postnatal day 15 in the pups, they observed increased aspartate content and the decreased gamma aminobutyric acid content in the hypothalamus of male offspring (Carbone et al. 2012). Human studies have not assessed neuroendocrine function, but the literature shows that higher phthalate metabolite concentrations in urine samples of pregnant women were associated with abnormal neurodevelopment of their children attesting to their central nervous system actions (Engel et al. 2009; Kim et al. 2011; Kobrosly et al. 2014).

The goal of the current study was to determine the effects on expression of neuroendocrine genes in the rats those prenatally exposure to DEHP from gestational day 14–19, a critical period of hypothalamic cell migration and targeting, during which sex differences in gonadal hormones organize hypothalamic nuclei (Maggi et al. 2014; Markakis and Swanson 1997). We focused on the immediate outcomes at birth and the long-term influences in adult rats. In adult offspring, experiments were conducted on three hormone-sensitive regions, the anteroventral periventricular nucleus (AVPV), the medial preoptic nucleus (MPN) and the arcuate nucleus (ARC) (Clarkson and Herbison 2009; Gore 2008; Wu and Gore 2010). These regions are the key targets of EDC and all involve in reproductive regulation (Clarkson and Herbison 2009; Wu and Gore 2010).

Materials and methods

Animals and treatments

All animal procedures were operated according to the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of People's Republic of China. The protocol was approved by the Animal Ethical and Welfare Committee of Tianjin Medical University (TMUaMEC 2013008). Sprague–Dawley rats (8 weeks' old) used for breeding were purchased from HFK BIOSCIENCE CO., LTD (Beijing, China). All animals were housed (2–3 per cage) under photoperiod cycle (12 h light: 12 h dark, lights on 7:00 a.m.) at room temperature (22 ± 2 °C). Standard diet (Rat and Mouse Maintenance Diet 1022, HFK BIOSCIENCE CO., LTD) was used in this study and filtered tap water was available ad libitum.

Rats were handled daily to minimize stress and were allowed to acclimate for 1 week prior to mating. Dams were mated with sexually experienced male rats in a random rotation to avoid paternal bias. The day that a vaginal plug was identified was termed gestation day (G0); 32 pregnant rats were randomly assigned to four groups ($n = 8$ per group) and treated with corn oil (vehicle control), 2, 10 or 50 mg/kg DEHP (Alfa Aesar) by gavage from G14 to G19. The DEHP was prepared fresh daily throughout the treatment period. The dosing volume was 10 ml/kg day and was adjusted based on individual maternal weight changes. All dams were housed and treated individually until the day of delivery. Postnatal day 1 (PND 1) was determined by the birth of at least one pup, when the numbers of live offspring, referred to as the F1 generation, were counted and sex ratio was determined. After delivery, each litter was standardized to eight pups (four males and four females). From PND21, the pups were weaned and were housed four same-sex littermates per cage. Body weights and anogenital distance (AGD) were weekly recorded from birth till the day for euthanizing. AGD was the distance from the caudal base of the genital tubercle to the anterior aspect of the anus. AGD was measured using a digital caliper and was recorded blind to exposure group by the same technician. The ratio of AGD to the cube root of body weight (AGD index) was calculated to evaluate AGD (Dickerson et al. 2011).

Tissue collection

On PND1, one male neonatal rat per litter was randomly chosen to be humanely euthanized by decapitation during lights on between 9:00 a.m. and 11:00 a.m. The sex of each male pup was confirmed by examining the testes from the carcasses. Brains were quickly removed and the hypothalami were dissected with the help of a stereomicroscope. The dissection was made ventral to the thalamus, posterior to

the optic chiasm, anterior to the mammillary bodies, and demarcated laterally by the optic tracts. All the hypothalamic samples were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. On PND70, one male rat per litter was randomly chosen to be euthanized by decapitation between 9:00 a.m. and 11:00 a.m. Brains were removed quickly and were cut into 1 mm slices with stainless steel brain matrices on the ice. Based on The Rat Brain in Stereotaxic Coordinates (Paxinos 2006), bilateral micropunches (1.25 mm diameter) were taken from the AVPV, MPN and ARC and stored at −80 °C until RNA extraction. Reproductive organs were removed and the wet weight was quickly measured using an analytical balance. The ratio of organ wet weight to body weight (organ coefficient) was calculated to evaluate organ development.

A second set of male adult rats were perfused for immunohistochemistry experiments. Rats were deeply anesthetized with 300 µl/100 g 10% chloral hydrate and were checked for lack of reflexes. Through the transcardial pathway, rats were perfused by flushing with 0.9% normal saline (NS) for 1 min followed by 4% paraformaldehyde (PFA) (Alfa Aesar) containing 0.1 M phosphate buffer (PB) and 0.9% NS for 10 min. After perfusion, brains were removed and post-fixed in 4% PFA at 4 °C for 24 h, cryoprotected in 20% sucrose in 0.1 M PB at 4 °C for 24 h, and then stored in cryoprotection buffer with 30% sucrose and 30% glycol in 0.1 M PB at −20 °C. After fixation, each brain was blocked at the optic chiasm and mammillary bodies into three parts using the brain matrix. Coronal brain sections (40 µm thicknesses) containing hypothalamus were cut using a vibrating blade microtome (Leica VT 1000S) and stored in cryoprotection buffer at −20 °C for subsequent immunohistochemistry procedure.

RNA extraction and quantitative RT-qPCR

An established double detergent lysis buffer system was used for total RNA extraction from frozen tissues (Jakubowski et al. 1991; Walker et al. 2009). Briefly, samples were homogenized on ice in lysis buffer using a 22-gauge needle and 1 cc syringe. Cytoplasmic RNA was treated with proteinase K and extracted with phenol chloroform, precipitated in isopropanol, and washed in ethanol. The resuspended RNA was treated with TURBO DNA-*free*™ kit (Ambion) following the manufacturer's protocol to remove contaminating genomic DNA. All RNA samples were run on a Thermo Scientific NanoDrop 2000 to assess purity and concentration.

Hypothalami of neonatal rats and micropunches taken from AVPV, MPN and ARC of adult rats were used for quantitative real-time PCR. In each group, six RNA samples were randomly selected from different litters for qPCR profiling. Cytoplasmic RNA (150 ng) was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. We designed a customized neuroendocrinology TaqMan® Array Card (Applied Biosystems) to include 46 genes selected for known neuroendocrine functions plus two housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, *Gapdh* and *18s*); this platform was used to compare relative hypothalamic gene expression by qPCR as published (Dickerson et al. 2011; Walker et al. 2014; Yin et al. 2015a). Although this array utilizes qPCR methodology, we previously validated it against gene-by-gene qPCR and showed excellent correspondence of results (Walker et al. 2009). Taqman Universal PCR Master Mix (Applied Biosystems) was used and all procedures were carried out according to the manufacturer's protocol. Real-time PCR was run on an ABI 7900 real-time PCR machine and the parameters were set as the following: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expression of each gene was determined using the comparative Ct method (Schmittgen and Livak 2008).

Immunohistochemistry and confocal microscopy

Immunohistochemistry was performed in eight adult rats of each treatment group for ERα in the AVPV, MPN and ARC and for CYP19 in the AVPV and MPN. Two sections per nuclei were chosen from each animal. The section was approximately bregma 0.0 and 0.04 for AVPV, bregma −0.6 and −0.64 for MPN, and bregma −2.52 and −2.56 for ARC. First, the sections were washed in PBS (PH 7.4), followed by incubation for 1 h in 10% normal goat serum, 0.05% Triton-X and 3% BSA in PBS. Next, sections were incubated with rabbit anti-ERα (1:500, Merck Millipore) and rabbit anti-CYP19 (1:100, Santa Cruz) for 48 h at 4 °C in 10% normal goat serum and 0.05% Triton-X. Fluorescein-conjugated goat anti-rabbit (1:400, ZSGB-BIO, China) was used as second antibodies in 10% normal goat serum for 2 h. By omitting primary antibodies in control samples, the specificity was verified and no staining was observed.

Confocal laser scanning microscope was used to compare the density of ERα and CYP19. From the surface of each section, a stack of ten images (in a 9 µm thickness with an interval of 1 µm) was captured using a confocal microscope (OLYMPUS FV1000) with 40× magnification. The laser parameters were kept constant to avoid saturation of immunofluorescence. Using NIH Image J 1.46r software, the images were processed and analyzed for immunoreactive areas. The regions of interest (ROI) were chosen using three circles (40 µm in diameter) along the border in the AVPV and four circles (37.5 µm in diameter) in the ARC. Immunofluorescent signals of ERα were quantified using the optical dissecting method (Kermath et al. 2014). Two images were used and the objects that were observable in the first section

but not in the adjacent sections were counted. Five pairs of images from each animal were processed and analyzed. The ERα puncta were quantified as particles from the reference section and the adjacent section. A projected image from the paired images was created using the Z Project function. The density of ERα puncta was estimated as [(# particles in reference section − # particles in project image)/volume]. The areas of ROI and the distance between two sections were used to calculate the volume (Yin et al. 2015b). Immunofluorescent signals of CYP19 were quantified by measuring the optical density. Dark pixels representing CYP19 immunostaining in the ROI were quantified as integrated optical density (IOD). Densities of CYP19 expression in ROI were calculated as mean IOD/area.

Data analysis

SPSS (version 19.0) was used to analyze development data, TLDA gene expression data, and immunohistochemistry data. The outliers were detected by a Grubbs test and were eliminated from data analysis. Normality and homogeneity of variance for each dataset was tested. Repeated-measure ANOVA was used to evaluate significant differences between DEHP treatment groups for body weights and AGD index of adult rats. For other datasets those met the criteria, comparisons were made by one-way ANOVA followed by Tukey post hoc analysis with $P < 0.05$ considered statistically significant. In some cases, the data were transformed (natural log or square root) and reanalyzed. If the transformed data did not meet the criteria of statistical analysis by one-way ANOVA, they were analyzed using the nonparametric Kruskal–Wallis test followed by Mann–Whitney analysis between each group. For gene expression, all samples were normalized to *Gapdh* expression before statistical analysis. For each gene expression and protein IOD, data were calibrated to the mean level in vehicle control to analyze fold change.

Results

Phthalate exposure effects on pregnancy and birth outcomes

Body weight gain of the dams was checked from G14 to G19 and the day before delivery and no significant differences were observed among control and DEHP treatment groups (Table 1). Sex ratios of male to female offspring showed no significant difference among DEHP exposure groups and control group. The developmental data of neonatal male rats were measured on PND1 (Table 2). Birth weights of male rats were significantly lower in the 10 and 50 mg/kg DEHP treatment groups ($P < 0.05$). AGD of male rats were increased only in the 2 mg/kg DEHP group ($P < 0.05$).

Phthalate exposure effects on somatic and reproductive development

As shown in Fig. 1a, the significantly main effect of DEHP treatment on postnatal body weight gain was observed in male rats. Post hoc analysis showed that males treated with 10 mg/kg ($P < 0.05$) and 50 mg/kg DEHP ($P < 0.05$) were lighter than control animals before postnatal 8 weeks. No effects of prenatal DEHP treatment on postnatal AGD index were observed before weaned (Fig. 1b). Moreover, prenatal DEHP treatment also resulted in the effects on organ development. In 2 mg/kg DEHP group, the organ coefficient of prostate in male rats was higher than that in control group (Table 3, $P < 0.05$). Except prostate, no alterations were observed in the organ coefficients of testicle, epididymis, seminal vesicle, preputial glands and levator ani (Table 3).

Neuroendocrine gene expression in hypothalamus on PND1

According to the function, 46 target genes were classified into five groups. The classification was as follows: steroid-hormone-signaling genes, neurotransmission genes, neuropeptide-signaling genes, circadian-related genes and growth-related genes (Online Resource). In neonatal male rats, the

Table 2 Birth data of male rats on PND1 (mean ± SD)

DEHP (mg/kg day)	*n*	Birth weight (g)	AGD (mm)
0 (vehicle)	8	6.74 ± 1.27	4.48 ± 0.55
2	8	6.85 ± 1.13	4.80 ± 0.68*
10	8	6.05 ± 0.97*	4.34 ± 0.66
50	8	6.29 ± 1.22*	4.32 ± 0.61

*$P < 0.05$ vs. vehicle

Table 1 Body weight data of dams (mean ± SD)

DEHP (mg/kg day)	*n*	Weight gain G14–19 (g)	Weight before delivery (g)
0 (vehicle)	8	49.53 ± 19.21	392.36 ± 39.43
2	8	53.00 ± 17.05	405.64 ± 54.23
10	8	47.50 ± 14.22	364.46 ± 62.82
50	8	41.21 ± 14.25	370.53 ± 53.32

genes were analyzed in the whole hypothalamus, and 11 genes in four groups were down-regulated (Table 4). Respectively, prenatal DEHP exposure down-regulated two genes involved in steroid hormone signaling (*Esr2*, *Cyp19a1*), one gene involved in neurotransmitter signaling (*Grin2a*), three genes involved in neuropeptide signaling (*Avpr1a*, *Kiss1r*, *Tac3r*), and five genes involved in regulation of biological rhythm (*Arntl*, *Clock*, *Dbp*, *Mtnr1a*, *Per2*). Results also show non-monotonic dose–response relationships in the 11 affected genes. Overall, the 10 mg/kg DEHP dosage was associated with the greatest number of genes expression changes in males.

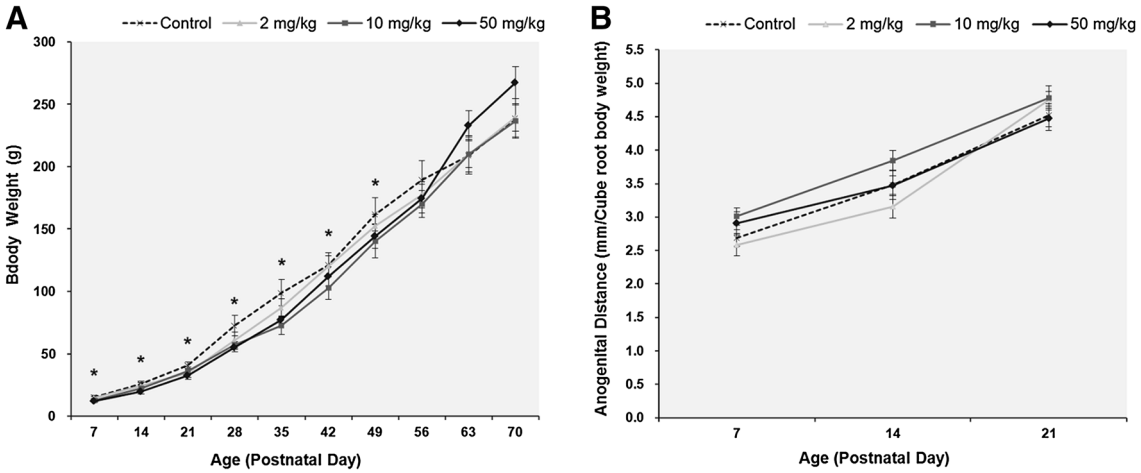

Fig. 1 Postnatal body weights and AGD index in four treatment groups. **a** Body weights; **b** AGD index. Data shown are mean ± SE. *$P < 0.05$ vs. vehicle

Table 3 Organ coefficient in male rats (%, $n = 8$, mean ± SD)

DEHP (mg/kg day)	Testicle	Epididymis	Seminal vesicle	Preputial glands	Prostate	Levator ani
0 (vehicle)	1.24 ± 0.11	0.37 ± 0.07	0.33 ± 0.08	0.07 ± 0.02	0.10 ± 0.04	0.32 ± 0.04
2	1.31 ± 0.09	0.39 ± 0.05	0.41 ± 0.07	0.06 ± 0.02	0.16 ± 0.04*	0.36 ± 0.06
10	1.24 ± 0.08	0.38 ± 0.04	0.34 ± 0.09	0.06 ± 0.01	0.12 ± 0.02	0.36 ± 0.07
50	1.27 ± 0.13	0.38 ± 0.07	0.33 ± 0.08	0.07 ± 0.01	0.11 ± 0.02	0.33 ± 0.06

*$P < 0.05$ vs. vehicle

Table 4 Relative expressions of altered genes in the hypothalamus of neonatal male rats (mean ± SE) (*n*)

Gene	DEHP (mg/kg day)				*F/H*	*P*
	0 (vehicle)	2	10	50		
Esr2	1.00 ± 0.14 (6)	0.56 ± 0.11* (6)	0.44 ± 0.09* (6)	0.76 ± 0.09 (5)	4.987	0.010
Cyp19a1	1.00 ± 0.27 (6)	0.48 ± 0.07 (5)	0.28 ± 0.03* (5)	0.65 ± 0.12 (6)	8.513	0.037
Grin2a	1.00 ± 0.18 (6)	0.64 ± 0.08 (5)	0.48 ± 0.03* (5)	0.96 ± 0.22 (6)	8.550	0.036
Avpr1a	1.00 ± 0.35 (6)	0.88 ± 0.22 (6)	0.21 ± 0.02* (5)	0.70 ± 0.17 (6)	11.402	0.010
Kiss1r	1.00 ± 0.08 (6)	0.76 ± 0.08 (6)	0.45 ± 0.06* (5)	1.11 ± 0.35 (6)	10.428	0.015
Tac3r	1.00 ± 0.21 (6)	0.52 ± 0.12 (5)	0.14 ± 0.04* (5)	0.69 ± 0.07 (6)	12.202	0.007
Arntl	1.00 ± 0.13 (6)	0.80 ± 0.10 (6)	0.28 ± 0.06* (5)	0.70 ± 0.07* (6)	8.633	0.001
Clock	1.00 ± 0.14 (6)	0.62 ± 0.04* (5)	0.55 ± 0.13* (5)	0.79 ± 0.09 (6)	3.252	0.046
Dbp	1.00 ± 0.16 (6)	0.55 ± 0.07* (5)	0.55 ± 0.12* (5)	0.76 ± 0.10 (6)	3.252	0.046
Mtnr1a	1.00 ± 0.20 (6)	0.68 ± 0.11 (6)	0.33 ± 0.08* (6)	0.50 ± 0.11* (5)	4.683	0.013
Per2	1.00 ± 0.19 (6)	0.68 ± 0.10 (6)	0.41 ± 0.12* (6)	0.77 ± 0.09 (5)	3.523	0.035

*$P < 0.05$ vs. vehicle

Neuroendocrine gene expression in the AVPV, MPN and ARC on PND70

In the hypothalamus of adult male rats, 46 target genes were respectively analyzed in the AVPV, MPN and ARC. In detail, prenatal DEHP exposure altered two genes in the AVPV (*Crhr1*, *Drd2*), three genes in the MPN (*Avp*, *Hcrtr2*, *Tac3r*), and nine genes in the ARC (*Avp*, *Esr1*, *Esr2*, *Ghrh*, *Kiss1*, *Npy*, *Pomc*, *Tac2*, *Trh*) (Table 5). Most of the affected genes were involved with steroid hormone signaling, neuropeptide signaling and growth. The 50 mg/kg DEHP treatment showed significant effects on gene expression in the MPN and ARC, which was clearly different from the results in neonatal hypothalamus. In addition, we also observed the enhanced expression of some genes in the AVPV, MPN and ARC.

Expression of ERα, CYP19 in the AVPV, MPN and ARC

In the AVPV, MPN and ARC of male offspring, ERα immunofluorescence was detected. The significantly fewer ERα expressions in the 50 mg/kg DEHP group than those of control rats were observed in the ARC (Fig. 2), but no alterations of ERα expression were found in the AVPV and MPN. Confocal microscopic images of ERα in the ARC are shown in Fig. 3. For CYP19, the immunofluorescence was detected in the AVPV and MPN of male rats and no significant differences of CYP19 density were observed in the AVPV and MPN.

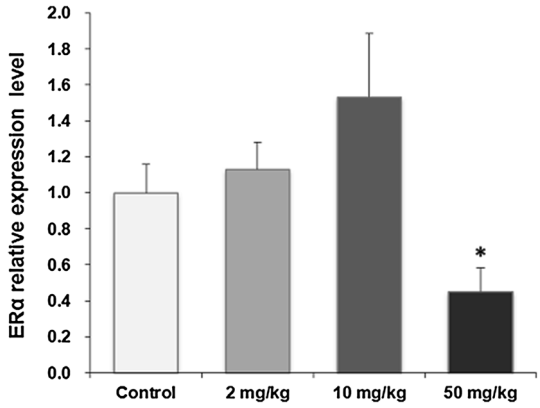

Fig. 2 ERα relative density in male ARC

Discussion

Human and animal studies have shown that the developmental toxicity of DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) is due to their capability of crossing the placental barrier (Axelsson et al. 2015; Do et al. 2012; EURAR 2008). This is highly relevant to the developing fetal hypothalamus, which is sensitive to both natural hormones and EDCs, and undergoes substantial organizational change during the period of sexual differentiation, setting the stage for sex-typical neuroendocrine functions that are manifested later in life (Gore et al. 2006; Kay et al. 2013). Our study reinforces this literature by showing that prenatal DEHP exposure has specific effects on development

Table 5 Relative expressions of altered genes in three hypothalamic nuclei regions of adult male rats (mean ± SE) (*n*)

Nuclei region	Gene	DEHP (mg/kg day)				*F/H*	*P*
		0 (vehicle)	2	10	50		
AVPV	*Crhr1*	1.00 ± 0.09 (6)	1.48 ± 0.14* (6)	0.95 ± 0.07 (6)	1.18 ± 0.13 (6)	4.562	0.014
	Drd2	1.00 ± 0.08 (5)	2.03 ± 0.29* (5)	1.16 ± 0.21 (6)	1.48 ± 0.24 (6)	3.950	0.025
MPN	*Avp*	1.00 ± 0.20 (6)	0.56 ± 0.12 (5)	0.25 ± 0.03* (6)	0.28 ± 0.08* (6)	6.836	0.003
	Hcrtr2	1.00 ± 0.15 (6)	1.24 ± 0.12 (6)	0.92 ± 0.05 (5)	1.72 ± 0.26* (6)	4.124	0.021
	Tac3r	1.00 ± 0.19 (6)	0.75 ± 0.14 (5)	0.41 ± 0.01* (5)	0.61 ± 0.07 (6)	9.775	0.021
ARC	*Avp*	1.00 ± 0.26 (5)	5.61 ± 3.89 (5)	1.63 ± 0.53 (6)	13.47 ± 5.56* (6)	8.704	0.033
	Esr1	1.00 ± 0.01 (5)	0.90 ± 0.06 (6)	0.87 ± 0.11 (6)	0.55 ± 0.04* (5)	10.273	0.016
	Esr2	1.00 ± 0.04 (5)	0.80 ± 0.08 (5)	1.13 ± 0.18 (6)	0.66 ± 0.05* (6)	11.128	0.011
	Ghrh	1.00 ± 0.14 (6)	0.97 ± 0.27 (6)	0.79 ± 0.15 (6)	0.35 ± 0.05* (5)	8.120	0.044
	Kiss1	1.00 ± 0.12 (6)	0.74 ± 0.12 (6)	0.72 ± 0.17 (6)	0.39 ± 0.03* (5)	9.018	0.029
	Npy	1.00 ± 0.17 (6)	0.58 ± 0.10 (6)	0.46 ± 0.09* (6)	0.24 ± 0.03* (5)	13.217	0.004
	Pomc	1.00 ± 0.07 (5)	0.52 ± 0.09* (5)	0.56 ± 0.15* (6)	0.33 ± 0.04* (5)	6.955	0.003
	Tac2	1.00 ± 0.13 (6)	0.85 ± 0.14 (6)	0.71 ± 0.10 (6)	0.51 ± 0.04* (5)	9.301	0.026
	Trh	1.00 ± 0.17 (5)	1.39 ± 0.28 (5)	1.86 ± 0.12* (5)	1.97 ± 0.21* (6)	4.754	0.014

*$P < 0.05$ vs. vehicle

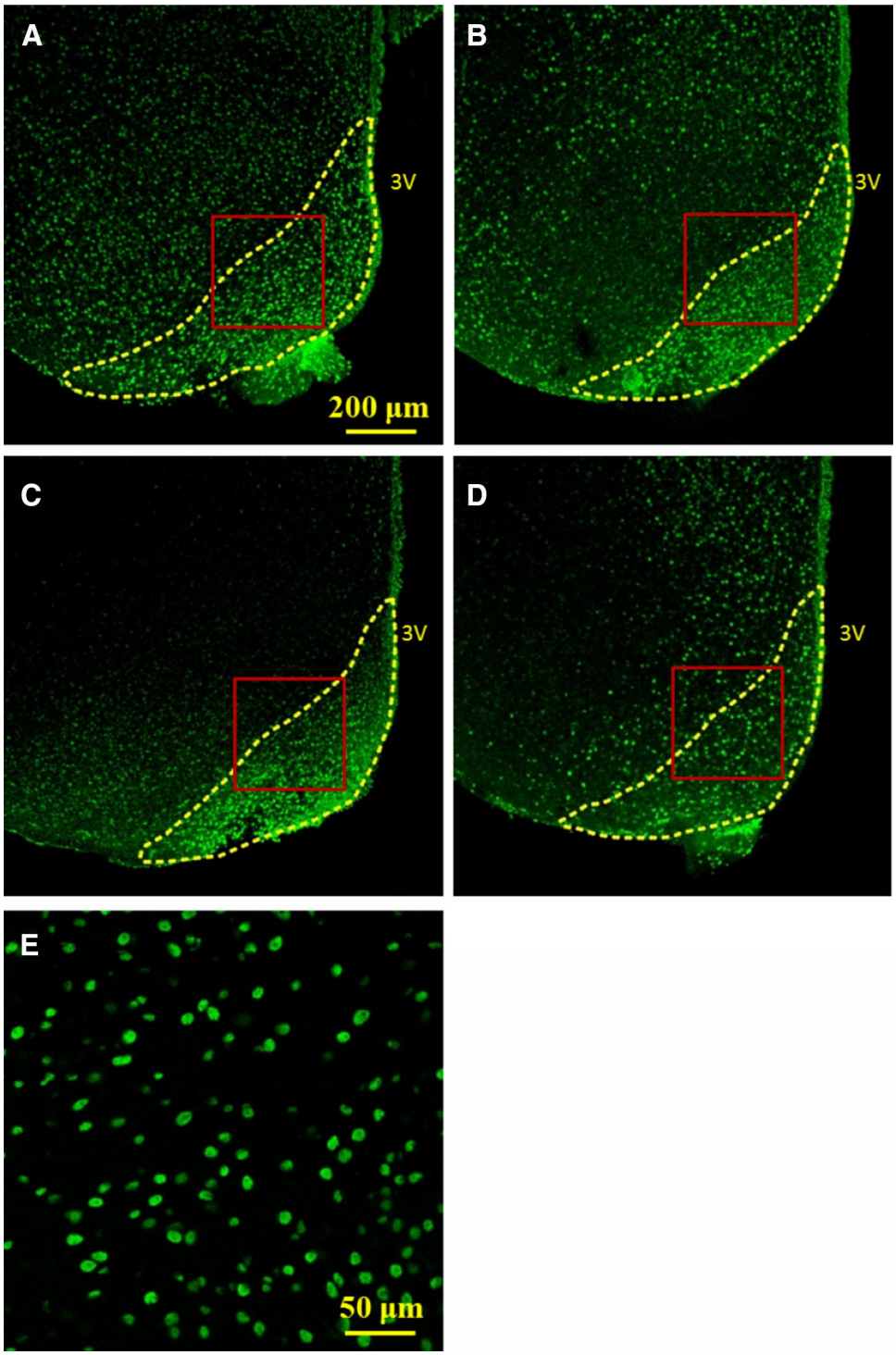

Fig. 3 Confocal microscopic images (×10) show estrogen receptor α (ERα) in the ARC (*outlined in yellow*) of male rats exposed to control vehicle (**a**), 2 mg/kg (**b**), 10 mg/kg (**c**), and 50 mg/kg DEHP (**d**). Quantification and analysis was performed at high-power magnification (×40) in the region *outlined in red* (**e**). *Scale bar* (*shown in panel* **a**, applies to *panels* **a**–**d**) = 200 µm. *Scale bar E* = 50 µm. 3 V, third ventricle (color figure online)

and hypothalamic neuroendocrine gene expression both in neonatal and adult male rats. The fact that DEHP is anti-androgenic may explain our results (Christiansen et al. 2010; Jarfelt et al. 2005), as male rats have much higher testosterone concentrations during prenatal life, blockade of which would be predicted to be disruptive in males.

The dosages of DEHP treatment from 2 to 50 mg/kg used in our study are considered relatively low. In the EU Risk Assessment Report of DEHP, the regulatory "no observed adverse effect level" (NOAEL) was 4.8 mg/kg for developmental toxicity in rats (EURAR 2008). The USA Center for the evaluation of risk to human reproduction (CERHR) also

published a NOAEL of DEHP on reproductive and development toxicity of between 1 and 10 mg/kg for oral exposure in rats (Kavlock et al. 2006). Human exposure to DEHP is regulated. The European Food Safety Authority (EFSA) established the tolerable daily intake (TDI) value for DEHP at about 50 μg/kg day (EFSA 2005). In a study on dietary exposure to phthalates in Belgium, 99% of DEHP intake distribution for preschool children in the worst case was equal to 80% of the TDI (Sioen et al. 2012). In another human monitoring study by Wittassek et al., the median daily intake of DEHP was about 2.4 μg/kg day in a German population between 1996 and 2003 (Wittassek et al. 2007). Thus, the dosage used in our study was high compared with human exposure, but relatively low compared with other reproductive and developmental toxicity studies on DEHP. It should be noted that dams in our study were fed with a standard diet, meaning that the effect of prenatal DEHP treatment occurs in conjunction with dietary phytoestrogens (Kanno et al. 2002).

Prenatal DEHP exposure interferes with somatic and reproductive development

In this study we observed a significant reduction in body weight due to prenatal exposure to the higher dosages of DEHP, and the inhibiting effects lasted into adulthood. Studies have shown that perinatal DEHP exposure at higher doses (300, 600 and 900 mg/kg) or lower doses (1.25 and 6.25 mg/kg) reduced birth weight of offspring rats (Christiansen et al. 2010; Lin et al. 2011). Our results are consistent with these published studies. Evidence suggests that exposure to EDCs during critical developmental period may alter energy metabolism and body weight (Fudvoye et al. 2014; Jin et al. 2014). Furthermore, we should note that the body weight of adult rats in 50 mg/kg DEHP group began to increase from postnatal 7 weeks. Our primary objective of this study was to investigate the effects of prenatal DEHP exposure on hypothalamic neurodevelopment, the association between DEHP treatment and catch-up growth may be analyzed in further study.

To determine reproductive developmental toxicity of EDCs, neonatal AGD of most mammals is a sensitive sexually dimorphic biomarker, as it is largely determined by androgen exposure during critical development periods (Hass et al. 2007; Hotchkiss et al. 2002; USEPA 1996). In our study, 2 mg/kg DEHP treatment increased neonatal AGD. Similarly, Do et al. found that exposure to 0.5, 1, 5, 500 and 50,000 μg/kg DEHP from gestational day 9–18 resulted in a tendency for increased AGD of male fetuses (Do et al. 2012). Other studies showed that perinatal exposure to 750 mg DEHP or 500 mg/kg DBP shortened AGD of male offspring (Moore et al. 2001; van den Driesche et al. 2011). In this study, postnatal AGD index before weaned were not affected. The disturbing effects of EDCs depend on exposure dosage, timing and duration, and prenatal DEHP exposure pattern in this study may be not able to influence AGD development after birth. Although AGD index was not affected, 2 mg/kg DEHP treatment stimulated the development of prostate in adult rats and indicated the potential reproductive toxicity in male offspring. Increasing evidence suggests that exposure to EDCs may have low-dose effects and non-monotonic dose–response (NMDR) relationships (Christiansen et al. 2010; Vandenberg et al. 2012), potentially explaining the influences of DEHP on reproductive development in our study.

Prenatal DEHP exposure alters neuroendocrine gene expression in hypothalamus

The hypothalamus is a heterogeneous and dynamically regulated network of dozens of different neural and glial cell types involved in the control of homeostasis. Because of the abundant expression of steroid hormone receptors, the hypothalamus is vulnerable to EDCs that interfere with sex hormone pathways (Gore 2010). Hypothalamic development depends on the presence of sex-appropriate timing and concentrations of steroid hormones during critical periods (Do et al. 2012; Fuchsl et al. 2013; Grande et al. 2006; Moore et al. 2001). However, little was known about DEHP effects on developing hypothalamic neuroendocrine systems. Our research focused on the male offspring treated by DEHP in utero and we identified 11 down-regulated genes in neonatal hypothalamus and 14 affected genes in adult AVPV, MPN and ARC. The results add to understanding the potential role of the hypothalamus in DEHP's long-lasting deficits in reproductive function and endocrine regulation, and add to prior research on hypothalamic effects of EDCs (Li et al. 2014; Walker et al. 2014; Wolstenholme et al. 2012).

DEHP down-regulated neuroendocrine genes expression in neonatal hypothalamus

For the offspring on PND1, the hypothalamus was still in development (Maggi et al. 2014). The whole hypothalamus was used in this study to detect neuroendocrine genes expression. Our results suggested that the reprogramming effects of prenatal DEHP exposure could be identified as early as the day after birth. In the fetal brain of male rats, high levels of testosterone produced from the testes act not only directly upon androgen receptor, but are also aromatized to estradiol (Dewing et al. 2003; Gore 2008; Wilson and Davies 2007). The *Cyp19a1* gene encodes cytochrome P450 aromatase, the key enzyme for estrogen biosynthesis in the hypothalamus (Roselli and Resko 2001). This gene contains response elements of both androgens and estrogens, and its expression is regulated by steroid hormones (Lephart

1996; Tabatadze et al. 2014), and it is sexually dimorphic (Lauber et al. 1997; Roselli and Resko 2001). Estrogen activity is mediated via the estrogen receptors α and β (ERα and ERβ), as well as membrane ERs. In the rat hypothalamus, while ERα is expressed more extensively than ERβ during the neonatal period and is thought to play important roles in sexual differentiation, ERβ has been speculated to be important in male during late gestation and at birth (Bakker et al. 2006; Cao and Patisaul 2011). Our evidence of down-regulated hypothalamic *Cyp19a1* and *Esr2* in male neonates indicates disruption of estradiol local synthesis and estrogen actions on its receptors. In addition, our data suggest that the genes related to neurotransmission, neuropeptide receptors and circadian regulation also are the targets of EDCs. We cannot draw specific conclusions regarding the degree to which hypothalamic functions could be altered with changes of these genes expression; however, our data support the possibility that these neuroendocrine genes could be altered in neonates after in utero exposure to DEHP. Moreover, most effects on gene expression were observed at the intermediate DEHP group (10 mg/kg) compared to the lower and higher dosages of DEHP groups (2 and 50 mg/kg, respectively). This type of NMDR relationship is both common and important in EDC toxicity studies (Vandenberg et al. 2012). Other DEHP toxicity studies also demonstrated NMDR relationships in testis Leydig cell dysfunction and in the inhibition of aromatase activity in the brain (Andrade et al. 2006; Ge et al. 2007). Our results may be helpful in guiding further DEHP toxicity studies especially in the animals during early life stage.

DEHP alters genes and protein expression with region-specific effects in adult hypothalamus

The reprogramming effects of DEHP treatment on neuroendocrine genes in three specific nuclei regions of adult offspring indicated the long-lasting effects of prenatal DEHP exposure on hypothalamic development. The AVPV and the MPN are two important sexually dimorphic regions in hypothalamus, which also are the targets of EDCs (Bateman and Patisaul 2008; Wu and Gore 2010). The AVPV of rats is smaller in male than female, and its function in male rodents is related to masculine sexual behavior and reproductive regulation (Bai et al. 2011; Davis et al. 1996; Gore 2008). In our study, gene expressions of *Crhr1* and *Drd2* in male AVPV were both up-regulated by prenatal DEHP treatment. These two genes encoded corticotropin-releasing hormone receptor 1 and dopamine 2 receptor, which both involved in the inhibitory actions on GnRH secretion (Jasoni et al. 2006; Liu and Herbison 2013; Takumi et al. 2012). This result may indicate the specific effects of prenatal DEHP exposure on neural networks in male AVPV, including aspects of the signaling pathways that regulate GnRH release. As the regulative center of sexual behavior, the MPN contains the sexually dimorphic nucleus of the preoptic area referred to as SDN-POA, which is larger in male than female rats (Gorski et al. 1980). In this study, we found three affected genes (*Avp*, *Hcrtr2* and *Tac3r*) in male MPN related to social behavior, food intake and reproduction (Bosch et al. 2010; Navarro et al. 2015; Scott et al. 2011), which indicated the differently possible impact of prenatal DEHP exposure on neuroendocrine regulation of MPN except for sexual behavior.

Except the AVPV and MPN, the ARC also is one of the targets of EDCs exposure on neurodevelopment (Gore et al. 2015; Walker et al. 2014). The ARC is involved in estrogen negative feedback on GnRH release, which needs the mediation of kisspeptin and neurokinin B (NKB) neurons, and suppression of kisspeptin and NKB system is in association with reduced GnRH secretion (Oakley et al. 2009; Rance et al. 2010). Our gene expression results showed four related genes (*Esr1*, *Esr2*, *Kiss1* and *Tac2*) were down-regulated. The decreased ERα protein expression also supported the inhibition effects of 50 mg/kg DEHP on estrogen action. It suggested that DEHP treatment may still interfere with GnRH release in male adult offspring via inhibiting kisspeptin and NKB action even the exposure occurred before their birth. Except for reproductive regulation, the ARC is a very important regulator of energy balance and appetite which is mainly mediated by NPY and POMC neurons (Sousa-Ferreira et al. 2014). The down-regulated expressions of *Npy*, *Pomc* and *Ghrh* in our study suggested that prenatal DEHP exposure may perturb the energy metabolism of adult rats by reprogramming hypothalamic neurodevelopment in utero. Besides, ARC Kisspeptin/Neurokinin B/Dynorphin neurons also take part in the action of estrogen on energy regulation (Mittelman-Smith et al. 2012). In general, the altered genes and protein in male ARC indicated a deeper insight on neuroendocrine development of prenatal DEHP exposure. Interestingly, our results in the ARC showed the dose–response relationships between DEHP treatment and genes expression, which may partly explain the lower body weight in higher dose of DEHP group. It indicated the regional and functional depended effects of prenatal DEHP exposure on the development of male offspring.

Conclusions

Our study provides additional evidence that in utero exposure to DEHP has a direct impact on male development of the neuroendocrine system. Specific effects on suites of neuroendocrine genes in the hypothalamus depend on the developmental stages. Moreover, the effects show non-monotonic dose–response relationships in neonatal male rats, with the intermediate dosage being associated with the greatest number of gene expression changes. On the contrary, the

effects on most of gene expressions pattern in adult male rats exhibit the linear dose–response relationships. By reprogramming the hypothalamus as early as the day after birth, the developmental trajectory of these animals is likely to be perturbed, with consequences for development, reproduction, energy metabolism, behaviors, and other functions controlled by the hypothalamus.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

Andrade AJM, Grande SW, Talsness CE, Grote K, Chahoud I (2006) A dose–response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose–response and low dose effects on rat brain aromatase activity. Toxicology 227(3):185–192. doi:10.1016/j.tox.2006.07.022

Araki A, Mitsui T, Miyashita C et al (2014) Association between maternal exposure to di(2-ethylhexyl) phthalate and reproductive hormone levels in fetal blood: the Hokkaido study on environment and children's health. PLoS One 9(10):2014. doi:10.1371/journal.pone.0109039 **(doi:ARTN e109039, eCollection 2014)**

Axelsson J, Rylander L, Rignell-Hydbom A, Lindh CH, Jonsson BA, Giwercman A (2015) Prenatal phthalate exposure and reproductive function in young men. Environ Res 138C:264–270. doi:10.1016/j.envres.2015.02.024

Bai YY, Chang F, Zhou R et al (2011) Increase of anteroventral periventricular kisspeptin neurons and generation of E2-induced LH-surge system in male rats exposed perinatally to environmental dose of bisphenol-A. Endocrinology 152(4):1562–1571. doi:10.1210/en.2010-1042

Bakker J, De Mees C, Douhard Q et al (2006) Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. Nat Neurosci 9(2):220–226. doi:10.1038/nn1624

Bateman HL, Patisaul HB (2008) Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus. Neurotoxicology 29(6):988–997. doi:10.1016/j.neuro.2008.06.008

Bosch OJ, Pfortsch J, Beiderbeck DI, Landgraf R, Neumann ID (2010) Maternal behaviour is associated with vasopressin release in the medial preoptic area and bed nucleus of the stria terminalis in the rat. J Neuroendocrinol 22(5):420–429. doi:10.1111/j.1365-2826.2010.01984.x

Cao JY, Patisaul HB (2011) Sexually dimorphic expression of hypothalamic estrogen receptors alpha and beta and Kiss1 in neonatal male and female rats. J Comp Neurol 519(15):2954–2977. doi:10.1002/cne.22648

Carbone S, Samaniego YA, Cutrera R et al (2012) Different effects by sex on hypothalamic–pituitary axis of prepubertal offspring rats produced by in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP). Neurotoxicology 33(1):78–84. doi:10.1016/j.neuro.2011.11.009

Christiansen S, Boberg J, Axelstad M et al (2010) Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. Reprod Toxicol 30(2):313–321. doi:10.1016/j.reprotox.2010.04.005

Clarkson J, Herbison AE (2009) Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinising hormone surge. J Neuroendocrinol 21(4):305–311. doi:10.1111/j.1365-2826.2009.01835.x

Davis EC, Shryne JE, Gorski RA (1996) Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. Neuroendocrinology 63(2):142–148

Dewing P, Shi T, Horvath S, Vilain E (2003) Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. Brain Res Mol Brain Res 118(1–2):82–90

Dickerson SM, Cunningham SL, Patisaul HB, Woller MJ, Gore AC (2011) Endocrine disruption of brain sexual differentiation by developmental PCB exposure. Endocrinology 152(2):581–594. doi:10.1210/en.2010-1103

Do RP, Stahlhut RW, Ponzi D, Vom Saal FS, Taylor JA (2012) Non-monotonic dose effects of in utero exposure to di(2-ethylhexyl) phthalate (DEHP) on testicular and serum testosterone and anogenital distance in male mouse fetuses. Reprod Toxicol 34(4):614–621. doi:10.1016/j.reprotox.2012.09.006

ECPI (2010) Plasticisers and flexible PVC information centre—plasticisers. http://www.plasticisers.org. Accessed 30 Mar 2015

EFSA (2005) Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. EFSA J 243:1–20

Engel SM, Zhu C, Berkowitz GS et al (2009) Prenatal phthalate exposure and performance on the Neonatal Behavioral Assessment Scale in a multiethnic birth cohort. Neurotoxicology 30(4):522–528. doi:10.1016/j.neuro.2009.04.001

EURAR (2008) European Union risk assessment report of bis(2-ethylhexyl) phthalate (DEHP). https://www.echa.europa.eu/documents/10162/e614617d-58e7-42d9-b7fb-d7bab8f26feb. Accessed 14 Sept 2015

Foster PM (2006) Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int J Androl 29(1):140–147. doi:10.1111/j.1365-2605.2005.00563.x **(discussion 181–185)**

Fuchsl AM, Langgartner D, Reber SO (2013) Mechanisms underlying the increased plasma ACTH levels in chronic psychosocially stressed male mice. PLoS One 8(12):e84161. doi:10.1371/journal.pone.0084161

Fudvoye J, Bourguignon JP, Parent AS (2014) Endocrine-disrupting chemicals and human growth and maturation: a focus on early critical windows of exposure. Vitam Horm 94:1–25. doi:10.1016/B978-0-12-800095-3.00001-8

Ge RS, Chen GR, Dong Q et al (2007) Biphasic effects of postnatal exposure to diethylhexylphthalate on the timing of puberty in male rats. J Androl 28(4):513–520. doi:10.2164/jandrol.106.001909

Gore AC (2001) Environmental toxicant effects on neuroendocrine function. Endocrine 14(2):235–246. doi:10.1385/ENDO:14:2:235

Gore AC (2008) Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. Front Neuroendocrinol 29(3):358–374. doi:10.1016/j.yfrne.2008.02.002

Gore AC (2010) Neuroendocrine targets of endocrine disruptors. Hormones 9(1):16–27

Gore AC, Heindel JJ, Zoeller RT (2006) Endocrine disruption for endocrinologists (and others). Endocrinology 147(6 Suppl):S1–S3. doi:10.1210/en.2005-1367

Gore AC, Walker DM, Zama AM, Armenti AE, Uzumcu M (2011) Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. Mol Endocrinol 25(12):2157–2168. doi:10.1210/me.2011-1210

Gore AC, Chappell VA, Fenton SE et al (2015) Executive summary to EDC-2: the endocrine society's second scientific statement on endocrine-disrupting chemicals. Endocr Rev. doi:10.1210/er.2015-1093

Gorski RA, Harlan RE, Jacobson CD, Shryne JE, Southam AM (1980) Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. J Comp Neurol 193(2):529–539. doi:10.1002/cne.901930214

Grande SW, Andrade AJ, Talsness CE, Grote K, Chahoud I (2006) A dose–response study following in utero and lactational exposure to di(2-ethylhexyl)phthalate: effects on female rat reproductive development. Toxicol Sci 91(1):247–254. doi:10.1093/toxsci/kfj128

Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L (2000) Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci 58(2):350–365

Guo Y, Kannan K (2011) Comparative assessment of human exposure to phthalate esters from house dust in China and the United States. Environ Sci Technol 45(8):3788–3794. doi:10.1021/es2002106

Hass U, Scholze M, Christiansen S et al (2007) Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environ Health Perspect 115(Suppl 1):122–128. doi:10.1289/ehp.9360

Hotchkiss AK, Ostby JS, Vandenburgh JG, Gray LE Jr (2002) Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley rat. Environ Health Perspect 110(Suppl 3):435–439

Jakubowski M, Blum M, Roberts JL (1991) Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. Endocrinology 128(6):2702–2708. doi:10.1210/endo-128-6-2702

Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H, Ladefoged O (2005) Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. Reprod Toxicol 19(4):505–515. doi:10.1016/j.reprotox.2004.11.005

Jasoni CL, Todman MG, Han S-K, Herbison AE (2006) Expression of mRNAs encoding receptors that mediate stress signals in gonadotropin-releasing hormone neurons of the mouse. Neuroendocrinology 82(5–6):320–328. doi:10.1159/000093155

Jin YX, Lin XJ, Miao WY et al (2014) Chronic exposure of mice to environmental endocrine-disrupting chemicals disturbs their energy metabolism. Toxicol Lett 225(3):392–400. doi:10.1016/j.toxlet.2014.01.006

Kanno J, Kato H, Iwata T, Inoue T (2002) Phytoestrogen-low diet for endocrine disruptor studies. J Agric Food Chem 50(13):3883–3885

Kavlock R, Barr D, Boekelheide K et al (2006) NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol 22(3):291–399

Kay VR, Chambers C, Foster WG (2013) Reproductive and developmental effects of phthalate diesters in females. Crit Rev Toxicol 43(3):200–219. doi:10.3109/10408444.2013.766149

Kermath BA, Riha PD, Woller MJ, Wolfe A, Gore AC (2014) Hypothalamic molecular changes underlying natural reproductive senescence in the female rat. Endocrinology 155(9):3597–3609. doi:10.1210/en.2014-1017

Kim SH, Chun S, Jang JY, Chae HD, Kim CH, Kang BM (2011) Increased plasma levels of phthalate esters in women with advanced-stage endometriosis: a prospective case-control study. Fertil Steril 95(1):357–359. doi:10.1016/j.fertnstert.2010.07.1059

Kobrosly RW, Evans S, Miodovnik A et al (2014) Prenatal phthalate exposures and neurobehavioral development scores in boys and girls at 6–10 years of age. Environ Health Perspect 122(5):521–528. doi:10.1289/Ehp.1307063

Lauber ME, Sarasin A, Lichtensteiger W (1997) Transient sex differences of aromatase (CYP19) mRNA expression in the developing rat brain. Neuroendocrinology 66(3):173–180

Lee HC, Yamanouchi K, Nishihara M (2006) Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behavior in rats. J Reprod Dev 52(3):343–352

Lephart ED (1996) A review of brain aromatase cytochrome P450. Brain Res Brain Res Rev 22(1):1–26

Li X, Jiang L, Cheng L, Chen H (2014) Dibutyl phthalate-induced neurotoxicity in the brain of immature and mature rat offspring. Brain Dev 36(8):653–660. doi:10.1016/j.braindev.2013.09.002

Lin Y, Wei J, Li Y et al (2011) Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat. Am J Physiol Endocrinol Metab 301(3):E527–E538. doi:10.1152/ajpendo.00233.2011

Liu X, Herbison AE (2013) Dopamine regulation of gonadotropin-releasing hormone neuron excitability in male and female mice. Endocrinology 154(1):340–350. doi:10.1210/en.2012-1602

Lomniczi A, Wright H, Castellano JM, Sonmez K, Ojeda SR (2013) A system biology approach to identify regulatory pathways underlying the neuroendocrine control of female puberty in rats and nonhuman primates. Horm Behav 64(2):175–186. doi:10.1016/j.yhbeh.2012.09.013

Maggi R, Zasso J, Conti L (2014) Neurodevelopmental origin and adult neurogenesis of the neuroendocrine hypothalamus. Front Cell Neurosci 8:440. doi:10.3389/fncel.2014.00440

Markakis EA, Swanson LW (1997) Spatiotemporal patterns of secretomotor neuron generation in the parvicellular neuroendocrine system. Brain Res Brain Res Rev 24(2–3):255–291

Mayer CM, Fick LJ, Gingerich S, Belsham DD (2009) Hypothalamic cell lines to investigate neuroendocrine control mechanisms. Front Neuroendocrinol 30(3):405–423. doi:10.1016/j.yfrne.2009.03.005

Mittelman-Smith MA, Williams H, Krajewski-Hall SJ et al (2012) Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology 153(6):2800–2812. doi:10.1210/en.2012-1045

Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE (2001) Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer di(2-ethylhexyl) phthalate. Environ Health Perspect 109(3):229–237

Navarro VM, Bosch MA, Leon S et al (2015) The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. Endocrinology 156(2):627–637. doi:10.1210/en.2014-1651

Oakley AE, Clifton DK, Steiner RA (2009) Kisspeptin signaling in the brain. Endocr Rev 30(6):713–743. doi:10.1210/er.2009-0005

Paxinos W (2006) The rat brain in stereotaxic coordinates, 6th edn. Elsevier, pp 126–186

Pocar P, Fiandanese N, Secchi C et al (2012) Exposure to di(2-ethylhexyl) phthalate (DEHP) in utero and during lactation causes long-term pituitary-gonadal axis disruption in male and female mouse offspring. Endocrinology 153(2):937–948. doi:10.1210/en.2011-1450

Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA (2010) Neurokinin B and the hypothalamic regulation of reproduction. Brain Res 1364:116–128. doi:10.1016/j.brainres.2010.08.059

Roselli CE, Resko JA (2001) Cytochrome P450 aromatase (CYP19) in the non-human primate brain: distribution, regulation, and functional significance. J Steroid Biochem Mol Biol 79(1–5):247–253

Schecter A, Lorber M, Guo Y et al (2013) Phthalate concentrations and dietary exposure from food purchased in New York State. Environ Health Perspect 121(4):473–494. doi:10.1289/ehp.1206367

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6):1101–1108

Scott MM, Marcus JN, Pettersen A et al (2011) Hcrtr1 and 2 signaling differentially regulates depression-like behaviors. Behav Brain Res 222(2):289–294. doi:10.1016/j.bbr.2011.02.044

Silva MJ, Barr DB, Reidy JA et al (2004) Urinary levels of seven phthalate metabolites in the US population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. Environ Health Perspect 112(3):331–338

Sioen I, Fierens T, Van Holderbeke M et al (2012) Phthalates dietary exposure and food sources for Belgian preschool children and adults. Environ Int 48:102–108. doi:10.1016/j.envint.2012.07.004

Sousa-Ferreira L, de Almeida LP, Cavadas C (2014) Role of hypothalamic neurogenesis in feeding regulation. Trends Endocrinol Metab TEM 25(2):80–88. doi:10.1016/j.tem.2013.10.005

Specht IO, Toft G, Hougaard KS et al (2014) Associations between serum phthalates and biomarkers of reproductive function in 589 adult men. Environ Int 66:146–156. doi:10.1016/j.envint.2014.02.002

Suzuki Y, Yoshinaga J, Mizumoto Y, Serizawa S, Shiraishi H (2012) Foetal exposure to phthalate esters and anogenital distance in male newborns. Int J Androl 35(3):236–244. doi:10.1111/j.1365-2605.2011.01190.x

Swan SH (2008) Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res 108(2):177–184. doi:10.1016/j.envres.2008.08.007

Swan SH, Main KM, Liu F et al (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113(8):1056–1061. doi:10.1289/ehp.8100

Tabatadze N, Sato SM, Woolley CS (2014) Quantitative analysis of long-form aromatase mRNA in the male and female rat brain. PLoS One 9(7):e100628. doi:10.1371/journal.pone.0100628

Takagi H, Shibutani M, Lee KY et al (2005) Impact of maternal dietary exposure to endocrine-acting chemicals on progesterone receptor expression in microdissected hypothalamic medial preoptic areas of rat offspring. Toxicol Appl Pharmacol 208(2):127–136. doi:10.1016/j.taap.2005.02.002

Takumi K, Iijima N, Higo S, Ozawa H (2012) Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. Neurosci Lett 531(1):40–45. doi:10.1016/j.neulet.2012.10.010

USEPA (1996) Guidelines for reproductive toxicity risk assessment. Fed Reg 61(212):56274–56322

van den Driesche S, Scott HM, MacLeod DJ, Fisken M, Walker M, Sharpe RM (2011) Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty. Int J Androl 34(6):E578–E586. doi:10.1111/j.1365-2605.2011.01175.x

Vandenberg LN, Colborn T, Hayes TB et al (2012) Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev 33(3):378–455. doi:10.1210/er.2011-1050

Walker DM, Juenger TE, Gore AC (2009) Developmental profiles of neuroendocrine gene expression in the preoptic area of male rats. Endocrinology 150(5):2308–2316. doi:10.1210/en.2008-1396

Walker DM, Goetz BM, Gore AC (2014) Dynamic postnatal developmental and sex-specific neuroendocrine effects of prenatal polychlorinated biphenyls in rats. Mol Endocrinol 28(1):99–115. doi:10.1210/me.2013-1270

Wilson CA, Davies DC (2007) The control of sexual differentiation of the reproductive system and brain. Reproduction 133(2):331–359. doi:10.1530/REP-06-0078

Wittassek M, Wiesmuller GA, Koch HM et al (2007) Internal phthalate exposure over the last two decades—a retrospective human biomonitoring study. Int J Hyg Environ Health 210(3–4):319–333. doi:10.1016/j.ijheh.2007.01.037

Wolstenholme JT, Edwards M, Shetty SRJ et al (2012) Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression. Endocrinology 153(8):3828–3838. doi:10.1210/En.2012-1195

Wu D, Gore AC (2010) Changes in androgen receptor, estrogen receptor alpha, and sexual behavior with aging and testosterone in male rats. Horm Behav 58(2):306–316. doi:10.1016/j.yhbeh.2010.03.001

Yin W, Maguire SM, Pham B et al (2015a) Testing the critical window hypothesis of timing and duration of estradiol treatment on hypothalamic gene networks in reproductively mature and aging female rats. Endocrinology 156(8):2918–2933. doi:10.1210/en.2015-1032

Yin W, Sun Z, Mendenhall JM et al (2015b) Expression of vesicular glutamate transporter 2 (vGluT2) on large dense-core vesicles within GnRH neuroterminals of aging female rats. PLoS One 10(6):e0129633. doi:10.1371/journal.pone.0129633