—Full Paper—

Effects of Perinatal Exposure to Phthalate/Adipate Esters on Hypothalamic Gene Expression and Sexual Behavior in Rats

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Abstract. Our previous research has identified the granulin (grn) and p130 genes as sex steroid-regulated genes in the neonatal rat hypothalamus that might be involved in sexual differentiation of the brain. Since phthalate/adipate esters such as di-n-butyl phthalate (DBP), diisononyl phthalate (DINP), and di-2-ethylhexyl adipate (DEHA) are suspected to interfere with the endocrine system as environmental endocrine disruptors having estrogenic or antiandrogenic properties, these chemicals may affect sexual differentiation of the brain. The present study assessed the effects of perinatal exposure to DBP, DINP, and DEHA on grn and p130 mRNA expressions in the hypothalamus on postnatal day (PND) 7 and sexual behaviors after maturation in rats. Maternal rats were given a phytoestrogen-free diet containing different doses of DBP (20, 200, 2,000, and 10,000 ppm), DINP (40, 400, 4,000, and 20,000 ppm) and DEHA (480, 2,400, and 12,000 ppm) from gestational day 15 to the day of weaning (PND 21). DBP and DINP exposure during the perinatal period resulted in an increase in hypothalamic grn and p130 mRNA levels in females and males, respectively, but DEHA exposure decreased expression levels of grn in males and p130 in females, although the effects were not dose-dependent. After maturation, male rats that were exposed to several doses of DBP, DINP, and DEHA displayed decreased copulatory behavior. The lordosis quotient was decreased in females perinatally exposed to DBP, DINP, and DEHA at all the doses used. On the other hand, serum levels of LH and FSH in both sexes and the estrous cycles in females were not affected by the treatments. These results suggest that inappropriate expression of grn and/or p130 genes in the brains of male and female neonatal rats by perinatal exposure to these chemicals may exert permanent effects on the hypothalamus, thereby decreasing sexual behavior after maturation.

Key words: Adipate, Granulin, Hypothalamus, p130, Phthalate, Sexual behavior

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In mammals, gonadal steroids play an essential role in the process of brain sexual differentiation during embryonic development and early postnatal life, which is known as the critical period. In this period, testosterone secreted from the testes in males acts to masculinize and defeminize the developing brain after conversion to estradiol by enzyme aromatase in the brain [1]. We

previously identified the granulin (grn) precursor gene [2] and p130 gene [3] as sex steroid-regulated genes in the rat hypothalamus during the critical period and suggested that these genes are involved in sexual differentiation of the rat brain [4-7]. According to our observations of the androgen- and estrogen-dependent induction of grn and p130 genes and of the sexually different patterns of their gene expression in the hypothalamus, it is possible that these genes are good parameters for assessing the sex steroid properties of environmental endocrine disrupting chemicals (EDCs) in the

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neonatal brain [5].

EDCs include a number of environmental chemicals that interact with the endocrine system, often due to their activity as a hormonal mimic [8]. The majority 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 [9, 10]. In recent years, phthalates have attracted much attention because several of these compounds are suspected of possess endocrine disrupting effects. Phthalate esters are used as plasticizers and are also found at low levels in foods, particularly fatty foods, such as dairy products, because they are fat-soluble [11, 12]. Di-n-butyl phthalate (DBP) initially attracted attention as a potential EDC because it was found to be a weak estrogen receptor agonist in some cell-based assays [13, 14]. Several studies have suggested that DBP may disrupt the endocrine system and produce marked alterations during male reproductive organ development [15–17]. The effects are also closely associated with the antiandrogenic activity produced by DBP in developing fetuses and newborns [18, 19]. In regard to diisononyl phthalate (DINP), the most commonly reported findings in rodents are liver and kidney enlargement and increased levels of peroxisomal enzymes [20], and thus, DINP is recognized to have peroxisome proliferator properties. In utero exposure to DBP [16, 21], DINP [22], and di-2-ethylhexyl phthalate (DEHP) [21, 23] has been shown to disrupt differentiation of androgen-dependent tissues in male rat offspring. Since some phthalates have testis toxicity and antiandrogenic effects, alternative compounds, such as di-2-ethylhexyl adipate (DEHA), have replaced phthalates as plasticizers in flexible polyvinyl chloride (PVC) products [24]. Although DEHA is currently being evaluated as a potential substitute for some phthalates, due to the similarities in structure and metabolism of DINP, DEHP, and DEHA, similarities in their actions may also exist.

In the present study, we assessed the effects of perinatal exposure to DBP, DINP, and DEHA on several aspects of reproductive function in rats, especially their effects on sexual differentiation of the brain. To this end, maternal rats were given a diet containing these chemicals from gestational

day 15 to the day of weaning, and anogenital distance (AGD) on postnatal day (PND) 1, serum sex-steroid hormone levels, and hypothalamic gene expression of *grn* and *p130* on PND 7, and sexual behaviors, gonadotropin levels, and estrous cyclicity after maturation were examined.

Materials 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 the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Agricultural and Life Sciences of the University of Tokyo. Wistar-Imamichi rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) that were approximately 8 weeks of age were kept in a room with controlled illumination (lights on 0500–1900) and temperature (23 ± 1 C), and provided chow and water *ad libitum*. Animals were mated (mating confirmed by the presence of sperm 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 PND 5. The dams were fed a 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 day of weaning (PND 21), the dams were provided a pulverized soy-free diet that contained 20, 200, 2,000, or 10,000 ppm of DBP, 40, 400, 4,000, or 20,000 ppm of DINP, or 480, 2,400, or 12,000 ppm of DEHA. After weaning, all the animals were fed a conventional pellet diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan).

Experimental design

On PND 1, body weight and AGD of pups were measured. On PND 7, pups were sacrificed by decapitation, and their brains were immediately removed. The entire hypothalamus, bordered

anteriorly by the optic chiasma, laterally by the hypothalamic fissures, and posteriorly by the mammillary body, was then dissected out [6]. Hypothalamic blocks were frozen in liquid nitrogen and stored at -80°C until RNA isolation. Trunk blood was taken and centrifuged at 3,000 rpm for 15 min at 4°C . The serum was collected and stored at -20°C until assayed for serum testosterone and estradiol concentrations. After maturation, estrous cyclicity of females was examined by daily microscopic observation of vaginal smears during postnatal weeks (PNW) 8-9 and PNW 19-20. To determine the preovulatory gonadotropin surge, blood samples were collected from tail veins without anesthesia at 1100 and 1600 h proestrus in female rats in PNW 20, and serum LH, FSH, and estradiol levels were measured. Blood samples were also taken from males in PNW 20 for measurement of serum LH, FSH, and testosterone levels.

In PNWs 20-21, copulatory behavior tests for both males and females were performed in a dark room at 1900 h, and were recorded with a VCR system (DCR-TRV20 digital video camera, Sony, Tokyo, Japan). In each behavioral test, a sexually inexperienced male was placed in a plastic cage ($40 \times 22 \times 18$ cm). After a 2 h adaptation period, a highly estrous ovariectomized female, primed with a subcutaneous estradiol silastic tube (70% cholesterol/30% 17β -estradiol) for 5 days and injected with 1 mg progesterone 6 h before the test, was introduced into the cage. Male sexual behavior was observed for 30 min after introduction of the female. The female was replaced by another similarly prepared female every 10 min to eliminate the influence of partner affinity. The following standard measures of male copulatory behavior were recorded: frequency of mount, intromission, and ejaculation during the 30 min; and interval between first ejaculation and subsequent mount (post-ejaculation interval, PEI). Regarding the frequency of mount and intromission, when the male ejaculated during the 30 min experimental period, the number of mounts and intromissions from the time of female introduction to the first ejaculation was converted into the number per 30 min. As for females, the lordosis reflex was examined on the day of proestrus by sexually experienced vasectomized-males. The lordosis quotient (LQ) was recorded as the number of lordosis reflexes/10 mounts by males $\times 100$ (%).

Real-time quantitative RT-PCR

Total RNA was isolated from the hypothalamus using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed into cDNA using a GeneAmp RNA PCR Kit (Applied Biosystems, NJ, USA) in a reaction volume of $20\ \mu\text{l}$ according to the manufacturer's protocol. RT-PCR was performed on a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH) according to the manufacturer's protocol. Primer sets were used for rat grn (forward primer, 5'-AGTTCTGAATGTCCTGACTCCGCCA-3'; reverse primer, 5'-AAGCCACTGCCCTGTTGGTCCTTT-3'), rat p130 (forward primer, 5'-GTGTAAGTGCTGCCTCGGGCACAG-3'; reverse primer, 5'-CGGGAACATGACCTGAGTATCTG-TTC-3') and rat 40S ribosomal protein S29 (RPS29) (forward primer, 5'-TGAAGGCAAGATGGGTCA-CCAGCAGC-3'; reverse primer, 5'-CAGGGTAGA-CAGTTGGTTTCATTGGG-3'). 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 s at 95°C for denaturing, 15 s at 60°C for annealing, and 15 s at 72°C for extension with 40 cycles. The cDNA was diluted one 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, Ann Arbor, MI, USA), respectively, according to the manufacturer's protocol. Serum concentrations of LH and FSH were measured by rat LH and FSH EIA systems (Amersham Pharmacia Biotech, Buckinghamshire, UK), respectively, according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were conducted using StatView (version J5, Abacus Concepts, Inc.). One-way analysis of variance and Dunnett's test were used to determine differences between the treated and control groups. Differences were considered

statistically significant at $P < 0.05$.

Results

Effects on body weight and AGD on PND 1

All the dams used delivered live pups. There were no significant differences in the number and sex ratio of pups per litter at birth among the groups. On PND 1, the body weight of the control males was significantly higher than the control females (Fig. 1A). In the DBP, DINP, and DEHA-exposed neonatal rats, body weights were significantly decreased compared with those of the control pups of the corresponding sexes, with the exception of the groups given 20 ppm of DBP for both sexes and 200 ppm of DBP for females. As shown in Fig. 1B, AGD in male neonates was decreased in a dose-dependent manner by maternal exposure to these chemicals. AGD in the 2,400 ppm and 12,000 ppm DEHA-exposed female neonates was also decreased. However, when AGD was normalized by the cube root of the body weight, exposure to DBP did not affect AGD in male neonates, although DINP at all doses used and the higher doses (2,400 and 12,000 ppm) of DEHA still decreased AGD in males (Fig. 1C). In female neonates, an increase in AGD was observed in DBP- and DINP-exposed animals at the highest doses, and the inhibitory effects of DEHA were no longer discernible.

Effects on serum sex steroid levels and hypothalamic gene expression on PND 7

In the control group on PND 7, serum testosterone levels were significantly higher in males than females (Fig. 2A), while there were no differences in serum estradiol levels between the sexes (Fig. 2B). Perinatal exposure to DBP, DINP, and DEHA did not affect the serum concentrations of testosterone and estradiol in either sex, with the exception that 2,000 ppm of DBP and 40 ppm of DINP significantly decreased estradiol levels in female pups. On PND 7, hypothalamic expression of *grn* mRNA was higher in males (Fig. 3A), and that of *p130* mRNA was higher in females (Fig. 3B). 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 was decreased by 480 and 12,000 ppm of DEHA. Hypothalamic

Fig. 1. Body weight (BW) (A), anogenital distance (AGD) (B), and AGD per cube root of BW ratio (C) in male and female rats on PND 1. Each column and vertical bar represents the mean $\pm$ SE (n=16-47). CO, control. *, $P < 0.05$ vs. CO of corresponding sex; †, $P < 0.05$ vs. male in CO group.

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.

Effects on sexual behavior and serum gonadotropin levels after maturation

Figure 4 shows the number of sexual behaviors in male rats examined in PNW 20-21. All of the control rats showed normal copulatory behaviors. Although the numbers of mounts and intromissions in the 40 ppm DINP- and 480 ppm DEHA-exposed rats were significantly decreased, perinatal exposure to DINP and DEHA at other doses and DBP at any dose did not affect the

Fig. 2. Serum levels of testosterone (A) and estradiol (B) in male and female rats on PND 7. Each column and vertical bar represents the mean $\pm$ SE ($n=5-7$). CO, control. *, $P<0.05$ vs. CO of corresponding sex; †, $P<0.05$ vs. male in CO group.

number of mounts or intromissions. On the other hand, a decrease in the number of ejaculations was observed in the 200 and 2,000 ppm DBP-, 40 ppm DINP-, and 48 and 12,000 ppm DEHA-exposed rats, while in the 10,000 ppm DBP-exposed rats, the number of ejaculations was increased. A decrease in PEI was observed only in the 10,000 ppm DBP-exposed rats. As shown in Fig. 5, serum LH, FSH, and testosterone levels in the male rats in PNW 20 were not affected by perinatal exposure to any of the chemicals used.

In both PNW 8-9 and 19-20, all control and chemical-exposed females had regular estrous cycles (data not shown). As shown in Fig. 6, the LQ of the females examined on the proestrous day in PNW 20-21 was significantly decreased in all the animals perinatally exposed to DBP, DINP, and

Fig. 3. Relative mRNA levels of grn (A) and p130 (B) in the hypothalamus of male and female rats on PND 7. Each value is normalized using RPS29. Each column and vertical bar represents the mean $\pm$ SE ($n=5-7$). CO, control. *, $P<0.05$ vs. CO of corresponding sex; †, $P<0.05$ vs. male in CO group.

DEHA at any dose. The inhibitory effect of the chemicals on female copulatory behavior was dose-dependent, except for DEHA. On the other hand, perinatal exposure to the chemicals did not affect serum levels of LH, FSH, and estradiol at either 1100 or 1600 h on the proestrous day in PNW 20 (Fig. 7).

Discussion

Previous studies have shown that in utero exposure to some phthalates produces a malformation of the male reproductive tract including a decrease in AGD, probably by reducing fetal testicular testosterone production [25-27]. It has been proposed that the relationship between

Fig. 4. The frequency of mount (A), intromission (B), ejaculation (C), and post-ejaculation interval (PEI) (D) in male rats in PNW 20. Each column and vertical bar represents the mean $\pm$ SE ($n=6-12$). CO, control. *, $P<0.05$ vs. CO.

AGD and body weight should be more properly evaluated using the cube root of the body weight [28, 29]. In the present study, perinatal exposure to DINP and DEHA indeed decreased AGD in a dose-dependent manner, even after normalization by the cube root of the body weight in male neonates, but the effect of DBP was no longer discernible. Since AGD is commonly regarded to be a measure of androgen-sensitive development in rodents [30], the antiandrogenic effect of DINP and DEHA appears to be more potent than that of DBP, at least under the present experimental conditions. Since there was no substantial change in serum testosterone and estradiol levels on PND 7, the inhibitory effect of the chemicals on fetal testicular testosterone production, if any, may be transient. Alternatively, DINP and DEHA might interact with androgen receptor as antagonists during the developmental period, and thus lead to a reduction in AGD. On the other hand, DBP and DINP at the

highest dose used increased AGD normalized by body weight in female neonates, suggesting a large amount of these phthalates may also have weak androgenic properties.

In the present study, the gene expression of *grn* in the hypothalamus was higher in males and that of *p130* was higher in females on PND 7, and this was consistent with our previous studies [2, 7]. Both DBP and DINP increased *grn* mRNA levels in female neonates, although not dose-dependently, and did not affect the levels in males. Phthalates may affect the expression of these genes by directly acting on the hypothalamus because they did not affect the serum sex steroid levels on PND 7. Several *in vitro* studies have shown that phthalates, such as DBP and butylbenzylphthalate (BBP), are capable of binding to estrogen receptor- α (ER α), inducing ER α -mediated gene expression, and enhancing the proliferation of MCF-7 human breast cancer cells, which express ER α [13, 14, 31, 32].

Fig. 5. Serum levels of LH (A), FSH (B), and testosterone (C) in male rats in PNW 20. Each column and vertical bar represents the mean $\pm$ SE (n=6-12).

Fig. 7. Serum levels of LH (A), FSH (B), and estradiol (C) in female rats at 1100 and 1600 h on the day of proestrus in PNW 20. Each column and vertical bar represents the mean $\pm$ SE (n=6-12). CO, control.

Fig. 6. The lordosis quotient of female rats in PNW 20. Each column and vertical bar represents the mean $\pm$ SE (n=6-12). CO, control. *, P<0.05 vs. CO.

Since we previously showed that androgen upregulates *grn* gene expression after conversion to estrogen [6], the increase in *grn* gene expression in the female hypothalamus may be due to the estrogenic properties of the phthalates. The reason why the effects of the chemicals were not dose-dependent is currently unclear, but a similar non-dose-dependent effect of DBP has been reported by others [33]. DBP and DINP increased p130 mRNA levels in only male neonates, though again not dose-dependently. The antiandrogenic 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. This adipate might exert antiandrogenic effects on males and androgenic effects on females, but

further studies are needed to clarify the precise mechanisms underlying the actions of phthalate/adipate esters on sex steroid-dependent gene expression in the hypothalamus.

After maturation, serum levels of LH, FSH, and testosterone in the male rats were unchanged by perinatal exposure to DBP, DINP, and DEHA, indicating that these chemicals do not affect the endocrine system of hypothalamo-pituitary-gonadal (HPG) axis in males. On the other hand, an inhibitory influence on male sexual behaviors was observed by perinatal exposure to these chemicals at several doses. The mount, intromission, and ejaculation frequencies were suppressed by the lowest doses of DINP and DEHA, and ejaculation was also suppressed by the highest dose of DEHA. The suppressive effect of DEHA on male sexual behavior might be due to the decrease in *grn* gene expression in the hypothalamus observed on PND 7 by DEHA, since a decrease in *grn* mRNA by infusion of an antisense oligodeoxynucleotide of *grn* gene into the third ventricle of male rats on PND 2 has been shown to result in a decrease in male sexual behavior after maturation [4]. Although the roles of p130 in the neonatal hypothalamus remain to be resolved, the suppressive effect of DINP on male sexual behavior might be related to an inappropriate increase in p130 gene expression in the neonatal hypothalamus. On the other hand, DBP at 200 and 2,000 ppm decreased ejaculation frequency, while it increased ejaculation frequency and shortened PEI at 10,000 ppm, without affecting the mount and intromission frequencies. As mentioned above, DBP at low doses increased p130 mRNA in the neonatal male hypothalamus, and at higher doses, increased *grn* mRNA in the neonatal female hypothalamus. Although not significantly, DBP at the highest dose also increased *grn* mRNA in the male hypothalamus. These changes in hypothalamic gene expression caused by perinatal exposure to DBP may account for its different effects on ejaculation after maturation. However, the possibility that other factors than *grn* and p130 are affected by perinatal exposure to the chemicals and involved in the decrease in sexual behavior in males after maturation cannot currently be ruled out.

In females, perinatal exposure to the chemicals used did not affect estrous cyclicity, serum estradiol levels, and gonadotropin surge on the day

of proestrous. This is consistent with recent studies by Mylchreest *et al.* [26] and Masutomi *et al.* [33] who observed that perinatal exposure to DBP or DINP did not affect estrous cyclicity after maturation. Recurrence of the estrous cycle is regarded as one of the manifestations of the sexual dimorphic functions of the brain, and perinatal administration of sex steroids induces persistent vaginal estrus associated with the lack of estrous cyclicity in rats [35]. Recurrence of the estrous cycle depends on a female-specific function of the HPG axis, namely, induction of a preovulatory surge of gonadotropin-releasing hormone (GnRH) and LH, which is induced by the positive feedback action of estrogen. It is therefore probable that sexual differentiation of brain controlling gonadotropin secretion is not affected by perinatal exposure to DBP, DINP, and DEHA.

Contrary to the endocrine system, lordosis, the posture adopted by the female rat in response to a mounting male during mating, was decreased by perinatal exposure to all the chemicals used at any dose. Since DBP and DINP increased *grn* gene expression and DEHA decreased p130 gene expression in the female hypothalamus, although not all at the doses, changes in the expression of these genes may account for the suppression of female sexual behavior. If this is the case, *grn* and p130 may be involved in the processes of not only masculinization (increase in male-type sexual behavior), but also defeminization (decrease in female-type sexual behavior) of the rat brain during the neonatal period. The brain region responsible for inducing preovulatory GnRH surge is thought to be the medial preoptic area (MPOA) [36] and that responsible for inducing lordosis is thought to be the ventromedial nucleus of the hypothalamus (VMH) [37], where sexually dimorphic pattern of the synaptic organization exists [38, 39]. Since we have shown that both *grn* and p130 genes are mainly expressed in the medial basal hypothalamus, including the VMH, the chemicals used in the present study may affect organization of the neuronal circuits in the VMH, but not the MPOA, by modulating expression of these genes during the processes of sexual differentiation of the brain.

In conclusion, the present study showed that inappropriate expression of *grn* and/or p130 genes in the hypothalamus of neonatal rats by perinatal exposure to DBP, DINP, and DEHA may result in a

decrease in sexual behavior after maturation in both sexes, without affecting the endocrine system of the HPG axis. These chemicals may act directly on discrete regions of the hypothalamus that regulate sexual behaviors, but not gonadotropin secretion, thereby affecting sexual differentiation of the brain with a resulting decrease in sex-specific behavior in adulthood.

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