Maternal Exposure to Dioxin Disrupts Gonadotropin Production
in Fetal Rats and Imprints Defects in Sexual Behavior

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; ER, estrogen receptor; GD, gestational day; CYP, cytochrome P450; StAR, steroidogenic acute-regulatory protein; LH, luteinizing hormone; eCG, equine chorionic gonadotropin; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; 8-Br-cAMP, 8-bromoadenosine-cAMP; FSH, follicle-stimulating hormone; ELISA, enzyme-linked immunosorbent assay; LHR, LH receptor; HSD, hydroxysteroid dehydrogenase; PND, postnatal day, AGD, anogenital distance; UVD, urethrovaginal distance AR, androgen receptor; GnRH, gonadotropin-releasing hormone; PK, protein kinase

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Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related substances are a class of environmental pollutants with suspected toxic effects on reproductive and developmental processes. This study investigated a hypothesis that maternal exposure to TCDD damages gonadotropin-regulated steroidogenesis in fetal gonads to imprint defects in sexual behavior as well as the maturation of gonadal tissues. Oral administration of 1 µg/kg TCDD to pregnant Wistar rats at gestational day (GD)15 attenuated the expression of luteinizing hormone (LH), a regulator of gonadal steroidogenesis, in the pituitaries of male and female fetuses at GD20. TCDD treatment also reduced the fetal expression of testicular and ovarian steroidogenic proteins including steroidogenic acute-regulatory protein. These changes in pituitary and gonadal proteins were fetus-specific, and this seems not to be due to the greater delivery of TCDD to the brain of fetuses than adults. This is because a reduction in LH production was not reproduced even although TCDD was administered intraventricularly to adult rats. Direct supplementation of equine chorionic gonadotropin (eCG), an LH-mimicking hormone, to TCDD-exposed fetuses at GD17 restored the reduced expression of gonadal steroidogenic proteins. Maternal exposure to TCDD delayed the development of gonadal tissues in male and female pups, and impaired their sexual behavior. However, eCG treatment at the fetal stage again restored not only tissue maturation but also many of the behavioral defects that appeared at adulthood. These results demonstrate that TCDD disrupts steroidogenesis in fetuses by targeting pituitary gonadotropin production, and imprints demasculinization in males and defeminization in females in terms of their copulatory behavior.
Introduction

Dioxins, exemplified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are a class of toxic environmental pollutants, and their harmful effects on ecological systems as well as humans continue to be of much concern (Schecter et al., 2006; Larsen, 2006). It is well known that dioxins produce a number of toxic effects in laboratory animals, such as wasting syndrome, immunotoxicity, and carcinogenesis, the mechanism of which is believed to involve activation of the aryl hydrocarbon receptor (AhR)(Poland and Knutson, 1982). However, TCDD-induced disorders in development and reproduction are potentially much more serious, because those injuries appear at lower dioxin doses (Peterson et al., 1993; Larsen, 2006). In utero and lactational exposure to TCDD leads to a number of disorders in the pups including retardation of growth, alteration of reproductive morphology, abnormal spermatogenesis, and defects in sexually-dimorphic behavior such as sexual behavior (Peterson et al., 1993). The mechanisms for these toxicities reported so far involve agonistic/antagonistic effects on estrogen receptor (ER)-dependent signaling (Ohtake et al., 2003), a reduction in the expression of sex steroid receptors (Ohtake et al., 2007), and induction of steroid-metabolizing enzymes (Badawi et al., 2000). Although the above mechanisms and their combinations would partially explain TCDD-induced reproductive toxicity, the exact mechanism remains largely unknown. Our previous studies have demonstrated that the treatment of pregnant rats with TCDD at gestational day (GD) 8 or 15 reduces the fetal expression of steroidogenic proteins including steroidogenic acute-regulatory protein (StAR) and cytochrome P450 (CYP) 17 (Mutoh et al., 2006; Takehoh et al., 2007): StAR is a transporter mediating the translocation of cytosolic cholesterol into inner mitochondria, a
rate-limiting process of steroidogenesis, and CYP17 is one of the obligatory enzymes producing both androgens and estrogens. These changes occurred at a TCDD dose of 1 µg/kg but not at 0.1 µg/kg, and in fetus- and testis-specific manners. Both of the latter effects are based on the observation that maternal exposure to TCDD causes a reduction in steroidogenic proteins in the fetal testis but not in the adrenals at GD20, and this defect disappears shortly after birth. Furthermore, it has been suggested that such defects produced by TCDD are due to a reduction in pituitary luteinizing hormone (LH). However, many matters remain to be clarified. For example, the following are still unknown: 1) the sex difference in the TCDD-produced damage on fetal steroidogenesis, 2) gonadotropin specificity, and 3) the mechanism for fetus-specific alterations.

In general, testosterone released from fetal gonadal tissues plays a crucial role in the acquisition of male and female sexual behavior in adulthood. For example, the traditional theory is that male development needs exposure of the developing brain to testosterone during a 'critical period', while female development requires either the absence of such stimulation or stimulation with lower concentrations of this androgen (MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; Döhler et al., 1984). More specifically, testosterone and/or the metabolites, 17β-estradiol and dihydrotestosterone, produced in the brain or peripheral tissues determine the direction of differentiation via switching masculinization and defeminization (Negri-Cesi et al., 2004; Carlson 2007). The steroid hormone-assisted differentiation includes a number of mechanisms which occur in a target gene-, exposing timing- or cell type-specific behavior (Davies and Wilkinson, 2006; Wilson and Davies, 2007). The 'critical period' differs among animal species, and it is the perinatal stage in rats (MacLusky and Naftolin, 1981;
Arnold and Gorski, 1984) although some claim that this period may be longer (Wilson and Davies, 2007). As mentioned above, we assume that the TCDD-produced reduction in circulating LH is a pre-requisite for the damage involving gonadal steroidogenesis during the perinatal stage. Taken together, it would be reasonable to assume that TCDD-induced LH reduction in a late fetal stage causes a disorder of sex-steroid biosyntheses, resulting in androgen/estrogen deficiency in the brain. If this is true, a reduction in pituitary LH will be a key mechanism for TCDD-induced reproductive toxicity, including sexually-dimorphic behavior. To assess the validity of this hypothesis, we examined the role of LH reduction during the fetal stage on gender-specific sexual behavior. This was investigated by examining whether direct supplementation of exogenous LH, as equine chorionic gonadotropin (eCG), to fetuses could rescue them from the damage caused by TCDD to sexual behavior at adulthood. In addition, the present study also focused on the sex difference in TCDD-produced damage to fetal gonadotropins and steroidogenesis, and on pathological development post-partum. We analyzed not only the differences but also the role of an LH reduction on these changes.
Methods

Materials

TCDD was purchased from Accu Standard Inc. (New Haven, CT, USA). The other reagents were of the highest grade commercially available.

Animals and treatments

All experiments were approved by the Institutional Animal Care and Experiment committee of Kyushu University. Female Wistar rats (7- to 8-weeks old) and male Wistar rats (9-weeks old) were purchased from Kyudo Co. Ltd. (Kumamoto, Japan), and these animals were bred by a standard procedure allowing them access to a commercial standard chow (CE-2, CREA Japan, Tokyo, Japan) and tap water ad libitum. They were kept in a room maintained at 21 ± 1°C and 50 ± 10% relative humidity under a 24 hr light/dark cycle (lit period: 07:00 - 19:00). Female rats were paired overnight with male rats. The next morning, sperm in the vaginal smears was checked to confirm pregnancy. When sperm was detected, the day was designated as GD0 of pregnancy. In all experiments, pregnant rats at GD15 were treated once with TCDD (1 µg/kg/2 ml corn oil, orally), and their fetuses were removed at GD20 for reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting analyses. Control dams were treated with corn oil alone. For the assessment of sexual behavior and pathological development, fetuses were allowed to be born and develop before the experiments. In some experiments, fetuses at GD17 were directly injected with eCG [5 I.U./5 µl phosphate-buffered saline (PBS)/body] or vehicle alone by the method reported.
previously (Mutoh et al., 2006). Briefly, fetuses in a dam anesthetized with pentobarbital were
given a single injection of eCG (all fetuses in the left uterus) or vehicle (all fetuses in the right
uterus) in their back, using a syringe fitted with a 31G needle. After the operation and
treatment, the wound of the dam was stitched up, and the pups were either removed at GD20
for the analyses of enzyme/hormone expression, or allowed to be born for the analyses of
sexual activity and pathological development. The data from the fetuses and born pups of one
dam were averaged to represent an analyzing unit. Therefore, all data represent the mean ±
S.D. of three or more parents.

In experiments using mature rats, male Wistar rats (7-weeks old) were treated orally (1
µg/kg/2 ml con oil x 1) or intra-ventricularly (10 µg/kg/25 µl corn oil x 1) with TCDD, and
the tissues were removed five days later. Control animals were treated with corn oil not
containing TCDD. The conditions of the intra-ventricular injection of drug dissolved in oil
were set by referring to the published procedures (Apostolakis et al., 1996).

Sexual behavior and saccharin preference

Male copulatory behavior was assessed twice at 10 and 13 weeks of age. The data at both
endpoints were virtually identical (data not shown), and male activity obtained at both 10 and
13 weeks of age was averaged to obtain a data unit. Adult female rats, sex partners, were
activated to be receptive according to the published procedures (Mably et al., 1992a). For this,
female rats were ovariectomized 2 weeks before the test (14 weeks of age at the operation),
and treated with 17β-estradiol (20 µg/0.1 ml corn oil, s.c.) and progesterone (1 mg/0.1 ml corn
oil, s.c.) 52 and 4 hr prior to the test, respectively. Before pairing with a test male, each female
was confirmed to be receptive by checking for both the presence of vaginal smears and hopping behavior toward a male which was different from a test male. The percentage of female receptivity attained by the above method was over 90%. A test male was paired with a receptive female for 30 min. Male-specific sexual behavior was evaluated using the following: the period until first mount and intromission (mount and intromission latency), and the number of mounts (mount frequency) and intromissions (intromission frequency). Female sexual behavior was assessed at 16 weeks of age. The females were activated to be receptive as described above. A test female was housed with a normal male for 30 min. Female-specific sexual behavior was assessed by observing the lordosis appearance per mount (lordosis quotient), and lordosis score ranging from 0 [none] to 3 [strong] (Hardy and Debold, 1972). All examinations for sexual behavior were carried out at during the period 21:00 - 24:00.

Saccharin preference in females was evaluated at 12 weeks of age without ovariectomy and steroid hormone treatment. For the first 4 days, the rats were given two bottles filled with tap water, and the daily water consumption was determined. On days 5 and 6, the rats were given a bottle containing tap water and a bottle containing 0.01% saccharin sodium salt. The amount of water consumption was determined by measuring the bottle weight per 24 hr. Saccharin preference was defined as the percentage of ingested volume of saccharin water vs. total water consumption.

**Organ culture**

Fetal testes and ovaries were removed from untreated pregnant rats at GD20. These tissues were cultured at 37°C in 5% CO₂ for 24 hr in the presence of either 100 nM TCDD, 1
mM 8-Br-cAMP, 10 I.U. eCG or their combination (Mutoh et al., 2006).

Reverse transcription-polymerase chain reaction (RT-PCR)

The extraction of mRNA, and RT-PCR was performed according to the method reported previously (Ishida et al., 2005). The cDNA amount-dependent amplification was achieved under the conditions described in the "(Supplemental Methods)". See also that section for primer designs.

Immunoblotting

Fetal testis (30 μl) and ovary (15 μl) were homogenized in an indicated volume of 5 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20% glycerol. They were then centrifuged at 1,000 x g for 10 min, and the supernatants were centrifuged at 9,000 x g for 20 min to prepare 9,000 x g supernatants. The resulting pellets were washed once with 0.2 ml homogenizing buffer, and suspended in PBS containing 5 mM EDTA and 1% nonidet P-40 to obtain mitochondrial fractions (10 μl/an ovary and 15 μl/a testis). The mitochondrial fractions were used for the immunoblotting of StAR and β-tubulin, and the 9,000 x g supernatants were used for CYP17 immunoblotting. To prepare fetal liver 9,000 x g supernatant, the tissue was homogenized in three volumes of the tissue weight of 10 mM Tris-HCl buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 1 mM PMSF, followed by centrifugation at 9000 x g for 20 min. Immunoblotting with anti-StAR, -CYP17, and -CYP1A1 antibodies was carried out by the methods described elsewhere (Ishida et al., 2005).
An antibody against rat steroidogenic acute-regulatory protein (StAR) was purchased from Abcam Ltd (Cambridge, UK). An antibody against guinea pig cytochrome P450 (CYP) 17 was a kind gift from Dr. S. Kominami (Graduate School of Integrated Arts and Sciences, Hiroshima University, Hiroshima 739-8521, Japan). An anti-CYP1A1 antibody was prepared in this laboratory.

Other methods

The fetal serum LH and follicle-stimulating hormone (FSH) were determined by ELISA using commercial kits (Endocrine Technologies, Inc., Newark, CA). The serum of male and female fetuses was diluted twice with diluents supplied with the kit before assay. The linearity of the calibration curve was confirmed over the range 0 to 12.5 ng LH or FSH/ml serum. The intra-assay variability was less than 8%. Statistical differences between two groups were examined by Student’s t-test. Statistical differences among multiple groups were compared by one-way ANOVA with a post-hoc test. In the frequencies of mount and intromission behaviors and lordosis quotient, the statistical difference was compared by the Mann-Whitney’s U-test after a Kruskal-Wallis test, because of their nature as non-consecutive variables.
Results

TCDD-produced change in gonadal steroidogenic proteins

Pregnant rats were treated with TCDD (1 µg/kg, p.o.) at GD15, and the expression of steroidogenic proteins in the fetal testis and ovary was determined at GD20. We firstly focused on two targets: StAR and CYP17. In male fetuses, the expression of testicular StAR and CYP17 was reduced by TCDD in terms of both mRNA and protein levels (Fig. 1, A and B). Also, in female fetuses, the expression of ovarian StAR mRNA was significantly reduced by TCDD (Fig. 1A). However, TCDD failed to reduce the expression of CYP17 in fetal ovaries (Fig. 1, A and B). These results suggest that maternal exposure to TCDD causes a decline in the expression of fetal steroidogenic protein(s) in both sexes although female CYP17 remained unchanged. As far as StAR and CYP17 are concerned, male fetuses seem to be more sensitive than female fetuses. Hepatic CYP1A1 was markedly induced by TCDD in both sexes, confirming TCDD transfer to fetuses from the dams (Fig. 1B).

To examine the possibility that TCDD reduces StAR expression through its direct effect on fetal gonads, testes and ovaries removed from untreated fetuses at GD20 were cultured with TCDD for 24 hr. If TCDD directly affects fetal gonads to reduce StAR, the same must be reproduced in cultured gonads treated with TCDD. However, TCDD did not reduce any expression of StAR mRNA (Fig. 2, A and B). The binding of LH to LH receptors (LHR) increases intracellular cAMP to enhance steroidogenesis. Indeed, 8-Br-cAMP, a permeable cAMP analogue, enhanced StAR expression in cultured fetal testis and ovary. However, TCDD again had no effect on cAMP-induced StAR expression (Fig. 2, A and B). Although
eCG induced StAR mRNA in cultured fetal testis, TCDD failed to affect this induction (Fig. 2A). In contrast to the testis, eCG did not produce a significant change in StAR expression in cultured fetal ovary (Fig. 2B). The results obtained indicate that TCDD reduces the expression of fetal StAR by a mechanism other than direct action on the gonads.

**TCDD-produced change in pituitary gonadotropins**

We then focused on the gonadotropins as the upstream regulator of gonadal steroidogenesis. Pregnant rats (GD15) were given oral TCDD, and the gonadotropin mRNAs and serum concentration of LH and FSH in female and male fetuses at GD20 were examined. As has been established, LH and FSH consist of two subunits, a common α-subunit and a specific β-subunit, whereas prolactin is a single subunit. The expression of LHβ and FSHβ mRNAs in both sexes was significantly reduced by TCDD, whereas prolactin mRNA was not altered following the treatment (Fig. 3A). Regarding α-subunit mRNA, it was significantly reduced only in males, although a trends toward a reduction was observed in females (Fig. 3A). In parallel with this, serum LH and FSH were also reduced by TCDD (Fig. 3B). These observations suggest that a reduction in LH/FSH production by TCDD in the fetal pituitary is the reason, at least in part, for the attenuated expression of steroidogenic proteins.

**Absence of TCDD-dependent attenuation in mature rats**

As mentioned in the Introduction, the reduction by TCDD in pituitary LH/FSH and testicular StAR is fetus-specific. Also, in the present study, when male 7-weeks old rats were given 1 µg/kg TCDD orally followed by mRNA analysis 5 days later, the pituitary LHβ/FSHβ
mRNAs were not changed in response to TCDD (Fig. 4A). It is conceivable that there is a threshold brain TCDD concentration needed for gonadotropin reduction and the transport of TCDD into the brain is far lower in mature rats than in fetuses. To investigate this hypothesis, young adult rats were given an intra-ventricular injection of 10 µg/kg TCDD. A separate experiment indicated that, under the conditions used, bromophenolblue diffused into the pituitary by way of the 3rd ventricle (photograph not shown). The observation that CYP1A1 mRNA in the pituitary was induced by TCDD supports the proposed targeting of TCDD (Fig. 4B). Even although TCDD was actually distributed to the pituitary and a very high dose of TCDD was used, neither pituitary LHß/FSHß mRNAs nor testicular StAR mRNA was reduced by TCDD (Fig. 4B). This evidence strongly suggests that the fetus-specific nature of the TCDD-produced attenuation of LH/FSH and StAR is due to a reason other than an age difference in the brain distribution of TCDD.

Recovery, by eCG from TCDD-induced defects in the expression of steroidogenic proteins.

If an LH/FSH reduction is indeed a critical requisite for a TCDD-produced reduction in the expression of steroidogenic proteins, supplying gonadotropins to TCDD-exposed fetuses should abolish the defects. To examine this possibility, we directly injected eCG, an LH-mimicking hormone, into the fetuses of pregnant rats (GD17) pre-treated with TCDD (GD15), and analyzed the steroidogenic genes in the fetal gonads at GD20 (Table 1). In addition to StAR and CYP1, the expression of fetal testicular CYP1A1, CYP19 and 3ß-hydroxysteroid dehydrogenase (HSD) was also reduced by TCDD. Although the same was observed in the female ovary, there were some differences between the sexes, for example,
17ß-HSD1 expression was reduced only in females, whereas CYP17 reduction occurred only in males. The number of PCR cycles needed for amplifying steroidogenic genes is much greater in males compared with females, except for a few cases such as CYP19 [see "(Supplemental table 1)"]. Thus, the TCDD-produced reduction in the expression of steroidogenic proteins seems to be greater and more serious in males than in females, even if the relative change compared with the control is similar in the two sexes. Receptors for sex-steroids, gonadotropins and TCDD did not exhibit any reduction by TCDD in both sexes (Table 1). Supplying eCG to fetuses almost entirely abolished the reduction by TCDD in the expression of gonadal steroidogenic genes (Table 1). This result strongly suggests that a TCDD-induced reduction in gonadotropins during fetal stages triggers the disorder of steroidogenesis in their gonads. Whether CYP19 expression in the brain is affected by TCDD is of interest, because 17ß-estradiol needed for brain development must be produced within this organ (see also Discussion). However, maternal exposure to TCDD did not cause any alteration in hypothalamic expression of CYP19 mRNA in male fetuses (data not shown).

Effect of fetal treatment with eCG on TCDD-induced retardation of tissue maturation.

Maternal treatment with TCDD (1 µg/kg) at either GD 8 or GD 15 did not cause a significant change in fetal body weight at GD20 in both sexes (data not shown). However, TCDD delayed their growth after birth, because the body weights of male pups at postnatal days (PND) 21 and 56 were significantly lower in pups born from dams given TCDD at GD15 than in the control (Table 2). With regard to gonadal tissues, a reduction in the weight of the ventral prostate was seen following maternal exposure to TCDD (Table 2). Although
the body weight of pups from TCDD-treated dams returned to the control level after PND70, a reduction in the weight of the ventral prostate was still seen at PND98. These defects in body/tissue weights were improved by giving the fetuses a single injection of eCG (Table 2). Also, in female pups, TCDD delayed the increase in body and ovary weights, and shortened anogenital and urethrovaginal distances (Table 2). However, as in the case of males, eCG treatment at fetal age restored or improved these disorders (Table 2). These observations suggest that TCDD disturbs the histological maturation of gender-specific tissues by reducing fetal gonadotropins.

Treatment of TCDD-exposed fetuses with eCG restores defects in sexual behaviors in adulthood.

It is of particular interest whether a fetus-specific reduction in gonadotropins followed by damage to steroidogenesis affects the development and/or acquisition of functional phenotypes in adulthood. We addressed this issue, and the pups born from dams treated with TCDD (GD15) were examined for their sexual activity in adulthood. In male pups, the latency until mount and intromission was markedly delayed and the mount and intromission frequencies were significantly reduced by maternal exposure to TCDD (Fig. 5, A-D). However, these abnormalities were restored by an injection of eCG into the fetuses, although the recovery in mount latency was incomplete (Fig. 5, A-D). Female pups born from TCDD-treated dams also exhibited significantly lower sexual activities, although the degree of impairment was much less than that seen in males, and the lordosis quotient was improved by injection of eCG into fetuses (Fig. 5, E and F). A preference for drinking water containing
saccharin, so-called 'saccharin preference', is one of the sexually-dimorphic forms of behavior (Valenstein et al., 1967). The saccharin preference in females was also reduced by TCDD, but recovered after eCG treatment (Fig. 5G). These results demonstrate that a TCDD-induced gonadotropin reduction at the fetal stage imprints disorders in sexually-dimorphic behavior in adulthood. The results also suggest that, as far as the damage caused by TCDD to sexual behavior is concerned, male pups are more sensitive than females.
Discussion

A number of epidemiological studies have suggested that there are reproduction- and development-related abnormalities in children born from parents exposed to dioxins (Schecter et al., 2006; Larsen, 2006; also see references therein). However, the mechanism remains largely unknown. This study showed that treatment of pregnant Wistar rats with a single oral dose of 1 µg/kg TCDD reduces the expression of gonadal steroidogenic proteins in female as well as male fetal rats. The dose used in this study seems to be far higher than the tolerable daily intake for humans (1-4 pg/kg/day) proposed by the WHO. However, such a comparison should be made with care. For example, because the body half-life of TCDD differs greatly between humans and rodents (humans >> rodents), it is thought that rodents need 100-times or more doses of dioxins, than humans, to suffer any harmful effects (Larsen, 2006). Therefore, it is suggested that the 'body burden' rather than the ingested dose is used for discussing dose-effect relationships in dioxin toxicology: the former is an index of the body content of dioxin corrected by the absorption rate and half-life as well as the amount ingested (Larsen, 2006). Faqi et al., (1998) have reported that there is a delay in sexual behavior in male Wistar rats following treatment of dams with TCDD at much lower doses than reported here. The reason for the inconsistency is unknown, but is discussed later in more detail. The body burden equivalent to the estimated human daily intake needed for causing the delay in male sexual behavior is predicted to be 9.5 pg/kg, based on the data of Faqi et al. (1998). Taking this value together with the daily intake of dioxins from foods (Domingo and Bacio, 2007) into consideration, reproductive and developmental disorders produced by dioxins
remain a matter which requires careful attention.

The present study did not analyze whether sex-steroids in the fetal rat testis are reduced by maternal treatment with TCDD. While some studies have reported that the testosterone level is not reduced (Haavisto et al., 2001; Timms et al., 2002), another study has claimed a decrease (Mably et al., 1992b). The reason for such inconsistency is unknown. All the above studies depend on commercial radioimmunoassay kits for testosterone analysis. One possibility is that these assays give false-negative/positive results due to the unidentified cross-reactivity of the antibody. The CYP17-mediated conversion of progesterone to the 17-hydroxylated metabolite is one of the steps necessary to produce androgens. In our previous studies, fetal testis removed from GD20 dams treated with TCDD at GD15 showed reduced activity (Mutoh et al., 2006; Taketoh et al., 2007). This evidence suggests that TCDD evokes a reduction in fetal testosterone, although this possibility needs to be clarified.

Based on the evidence that a TCDD-produced reduction in gonadal steroidogenesis is abolished by eCG supplementation to fetuses, it seems to largely depend on the reduced production of pituitary gonadotropins. It has been thought that the acquisition of masculine sexual behavior requires exposing the brain to testosterone and 17ß-estradiol at the 'critical period' (Negri-Cesi et al., 2004; Sato et al., 2004). This period is basically between 18 and 27 days after conception in rats (MacLusky and Naftolin, 1981; Arnold and Gorski, 1984), although it may continue until about one month after birth or more (Davies et al., 1995; Wilson and Davies, 2007). Although the male fetal brain needs estrogen as well as androgen to differentiate, serum 17ß-estradiol cannot enter the brain because α-fetoprotein, a binding protein with high affinity for this steroid, is present in fetal serum (Bakker and Baum, 2008
Therefore, 17β-estradiol needed for development must be synthesized from testosterone by CYP19 in the male fetal brain, although a portion of unbound 17β-estradiol in serum is translocated into the brain (Bakker and Baum, 2008). Thus, fetal brain depends on the testis for the supply of testosterone, an estrogen precursor, for its development. In connection with this issue, previous work has suggested that testosterone production in male fetal rats is independent of LH-mediated regulation until GD19.5 (Habert and Picon, 1982; El-Gehani et al., 1998). This is based on the evidence that, while fetal testicular testosterone appears at around GD15, and its concentration increases sharply at GD18, plasma LH does not appear until GD19.5. In the present study, we observed a concomitant reduction by TCDD in LH and gonadal steroidogenic proteins at GD20 without a reduction in LHR. Taking all above information into consideration, it would be reasonable to believe that the regulation of fetal testosterone formation by LH becomes functional at the final stages of gestation and this plays an important role in brain development. The observation obtained here, that giving an eCG supplement to TCDD-exposed fetuses restores not only a reduction in the testicular expression at GD20 of steroidogenic proteins but also defects in sexual behavior in adulthood, strongly supports the above view. In an LH-independent regulation of testosterone formation which operates before GD19.5, several peptides such as pituitary adenylate cyclase-stimulating polypeptide are suggested to play a role (El-Gehani et al., 2000; El-Gehani et al., 2001). Therefore, an alternative possibility is that exogenous eCG may compensate for the TCDD-produced defect in such peptides. However, it remains to be clarified whether TCDD has a substantial effect on non-LH regulators mentioned above.

The traditional concept is that feminization of females occurs in the absence of
stimulation with gonadal steroids (Negri-Cesi et al., 2004; Carlson 2007). With regard to this, although the fetal ovary is capable of producing testosterone, it has only a limited ability to secrete this androgen (Wilson et al., 1981). Our observation that a cultured ovary removed at GD20 does not respond to eCG is consistent with this, although a trend towards a positive response was observed (Fig. 2B). Nevertheless, eCG supplementation at GD17 to TCDD-exposed female fetuses improved the impaired expression of steroidogenic proteins as well as the disorders in sexual behavior. It has been demonstrated that feminizing differentiation needs estrogen at much lower concentrations than masculinization (Döhler et al., 1984). While ERα-deficient female mice lose their sexual receptivity and lordosis behavior (Ogawa et al., 1998), androgen receptor (AR)-knockout females exhibit normal activity (Sato et al., 2004). These findings support the view that estrogen-mediated signaling is one of the necessary factors for acquiring female phenotypes. Therefore, the partial restoration by eCG treatment of TCDD-produced damage to sexual behavior in females seems to be due, at least partially, to the improvement in ovarian steroidogenesis in the late gestational periods.

This study has shown that maternal exposure to TCDD causes reductions in the mount and intromission frequencies as well as their latency in male pups. However, this observation is partially inconsistent with previous reports. For example, earlier studies in rats have demonstrated that while TCDD extends the latency of mount, intromission and ejaculation, the frequencies of these behaviors are either increased or unchanged following prenatal treatment with TCDD. Those studies used Holtzman (Mably et al., 1992a; Bjerke et al., 1994), Long-Evans (Gray Jr. et al., 1995) and Wistar strains (Faqi et al., 1998). Thus, one of the
reasons for the discrepancy might be due to strain differences. The observation that the structures of the brain regions associated with sexual behavior differ among rat strains supports this view (Garcia et al., 2005). However, Faqi et al. (1998) have reported the lack of a TCDD effect on masculine behavioral frequency even in the Wistar strain which is the one used in the present work. Although the reason for the difference remains unclear, the TCDD dose in their study (chronic treatment from fetal to lactational stages) might be insufficient to adversely affect masculine behavior frequency. If this was true, behavioral latency would be the most-sensitive index for a dioxin-produced defect in copulatory capacity.

As shown in this study, TCDD reduces the syntheses of pituitary LHβ and FSHβ in male and female fetuses and the α-subunit in male fetuses without affecting the production of prolactin. This observation strongly suggests that TCDD impairs brain function in an LH/FSH-specific fashion. It is well known that gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus plays a crucial role in the synthesis of LH/FSH (Burger et al., 2004). However, the expression of hypothalamic GnRH and pituitary GnRH receptor mRNA in male and female fetuses remains unchanged by TCDD (data not shown). Thus, TCDD attenuates fetal LH/FSH by a mechanism different from a reduction in GnRH and its receptors. The intracellular mechanism underlying GnRH-dependent regulation of LHβ, FSHβ and the α-subunit is very complex. The binding of GnRH to its receptors activates multiple signal-transducing pathways including cAMP-protein kinase A (PKA), mitogen-activated protein kinase-mediated PKC and the intracellular Ca²⁺-dependent pathway (Burger et al., 2004). TCDD may affect these pathways to produce a reduction in LH/FSH production, although there is little evidence supporting this at present.
In conclusion, this study provides evidence that maternal exposure to TCDD imprints defects in gender-specific sexual behavior through down-regulation of gonadotropin production. Further studies are needed to clarify the detailed mechanism.
References


Gray Jr LE, Kelce WR, Monossen E, Ostby JS, and Birnbaum LS (1995) Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland


Mably TA, Moore RW, and Peterson RE (1992b) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin: 1. effects on androgenic status. *Toxicol Appl*


*Science* **211**: 1278-1284.
Footnotes

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Legends for Figures

Fig. 1: Effects of TCDD on the fetal expression of gonadal StAR and CYP17 mRNAs (A) and proteins (B). StAR/CYP17 mRNAs and the protein levels in fetal (GD20) testis and ovary were analyzed following maternal exposure (GD15) to TCDD. Each bar of panel A represents the means ± SD (N=3 dams; two fetuses/one dam were analyzed). *Significant difference between the pairs indicated (P < 0.05). NS: Not significant. In experiment (B), mitochondrial β-tubulin in gonadal tissues and hepatic microsomal CYP1A1 were also analyzed as a reference. The amount of protein loaded was 5 µg (male CYP17 and male/female β-tubulin) and 30 µg (male StAR and female CYP17), respectively. For immunoblotting of CYP1A1, 10 µg protein of hepatic 9,000 x g supernatant was electrophoresed. StAR protein was hardly detected in the fetal ovary (data not shown). Four fetal samples were prepared from each of the dams.

Fig. 2: Effects of TCDD on StAR expression in cultured fetal testis (A) and ovary (B). Testes and ovaries were removed from fetuses in untreated pregnant rats (GD20), and treated with indicated reagents for 24 hr, followed by measurement of StAR mRNA. See Materials and Methods for the details of cell treatment. Each bar represents the mean ± S.D. of 3 cultures. *Significant difference between the pairs indicated (P < 0.05). NS: Not significant.

Fig. 3: TCDD-induced alteration in the level of fetal gonadotropins in pituitary mRNA (A) and serum protein (B). Gonadotropin mRNAs in fetuses (GD20), the parents of which were
treated with TCDD at GD15, were determined by RT-PCR. Serum LH and FSH were analyzed by ELISA. Each bar in Panel A represents the mean value relative to the control ± S.D. (N=5 dams; two fetuses/one dam were analyzed). In Panel B, each bar also represents the value relative to the control ± S.D. [N=3 dams except for FSH (N=6 dams); two fetuses/one dam were analyzed]. Control values (ng/ml serum) were as follows: male LH and FSH were 0.96 ± 0.11 and 0.49 ± 0.08, respectively; female LH and FSH were 1.20 ± 0.12 and 1.19 ± 0.30, respectively. Abbreviations: αSub, common α-subunit of LH and FSH; and Prol, prolactin.

**Fig. 4:** Absence of attenuation of StAR and gonadotropins in male pubertal rats following oral administration (A) and intra-ventricular injection (B) of TCDD. In experiment A, male pubertal rats were treated once with 1 µg TCDD/kg/2 ml corn oil (p.o.), and pituitary mRNAs were analyzed 5 days after treatment. In B, the changes in mRNAs coding testicular StAR, and pituitary gonadotropins and CYP1A1 were determined 5 days after intra-ventricular injection of TCDD. In this experiment, TCDD (10 µg/kg/25 µl corn oil) was injected into the lateral ventricle (LV) at the position described below: antero-posterior, -0.8 mm; lateral, 1.0 mm; and vertical, 3.7 mm. Injection of solution into the LV and diffusion to other ventricles (3rd and 4th ventricles) was confirmed by the distribution of bromophenol blue (0.1% aqueous solution, injection volume, 5 µl) Each bar represents the mean (relative value to the control) ± S.D. of 4-5 rats. *Significant difference compared with the control (P < 0.05). NS: Not significant.
Fig. 5: Recovery following in utero injection of eCG from TCDD-induced defects in gender-specific sexual behavior and saccharin preference in females. Male and female pups born from dams treated with TCDD at GD15 were examined for their activity in adulthood. In males, latency until first mount (A) and the number of mounts (B), and latency until first intromission (C) and the number of intromissions (D) were observed after pairing with normal females ovary-excised and pre-activated with progesterone and 17β-estradiol (see Materials and Methods). In the case of females born from TCDD-treated dams, they were pre-activated as described above, and then coupled with normal males to examine the occurrence (E) and intensity (F) of lordosis behavior. Saccharin preference in females was examined without ovariectomy and progesterone/estrogen pre-treatments (G). In all experiments, eCG was directly injected into fetuses at GD 17. Bars are means ± S.D. (N=3 dams; at least two pups born from one dam were analyzed). *Significant difference (P < 0.05) between the pair indicated.
TABLE 1

Recovery of TCDD-produced decrease in fetal mRNAs by in utero injection of eCG

Each value represents the mean ± S.D. (N=3-4 dams; two fetuses/one dam were analyzed).

Pregnant rats were treated at GD15 with TCDD, and then their fetuses were treated either with PBS (TCDD + PBS) or eCG (TCDD + eCG) at GD17. In control, dams and the fetuses were treated with vehicle (corn oil) and PBS, respectively. The expression of mRNA was normalized by β-actin mRNA.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Male (Testis)</th>
<th>Female (Ovary)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCDD + PBS</td>
<td>TCDD + eCG</td>
</tr>
<tr>
<td>StAR</td>
<td>32 ± 11</td>
<td>108 ± 26</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>49 ± 10</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>CYP17</td>
<td>49 ± 14</td>
<td>103 ± 25</td>
</tr>
<tr>
<td>CYP19</td>
<td>51 ± 7</td>
<td>97 ± 28</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>49 ± 9</td>
<td>90 ± 14</td>
</tr>
<tr>
<td>17β-HSD1</td>
<td>74 ± 12</td>
<td>85 ± 38</td>
</tr>
<tr>
<td>17β-HSD3</td>
<td>81 ± 40</td>
<td>122 ± 24</td>
</tr>
<tr>
<td>AR</td>
<td>81 ± 29</td>
<td>75 ± 15</td>
</tr>
<tr>
<td>ERα</td>
<td>119 ± 9</td>
<td>118 ± 28</td>
</tr>
<tr>
<td>ERβ</td>
<td>104 ± 4</td>
<td>97 ± 16</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Basal</td>
<td>'TCDD + PBS'</td>
</tr>
<tr>
<td>--------------</td>
<td>-------</td>
<td>--------------</td>
</tr>
<tr>
<td>LHR</td>
<td>97 ± 16</td>
<td>105 ± 10</td>
</tr>
<tr>
<td>FSHR</td>
<td>93 ± 2</td>
<td>103 ± 29</td>
</tr>
<tr>
<td>AHR</td>
<td>95 ± 10</td>
<td>86 ± 27</td>
</tr>
<tr>
<td>ARNT</td>
<td>104 ± 4</td>
<td>98 ± 27</td>
</tr>
</tbody>
</table>

\(^a\) Significantly different from control (Corn oil + PBS) (P < 0.05)

\(^b\) Significantly different from 'TCDD + PBS' (P < 0.05)

N.D., not detectable.

Abbreviations: PBS, phosphate-buffered saline; HSD, hydroxysteroid dehydrogenase; AR, androgen receptor; ER, estrogen receptor; LHR, LH receptor; FSHR, FSH receptor; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator.
TABLE 2

Effect of direct injection of eCG into fetuses on TCDD-induced changes in the weights of body and reproductive organs, and in anogenital and urethrovaginal distances.

Each value represents the mean ± S.D. of 13-18 fetuses removed from 3-4 dams.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dam:</th>
<th>Control</th>
<th>TCDD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetus:</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Male pups</td>
<td></td>
<td>(N=18)</td>
<td>(N=16)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND21</td>
<td>54.3 ± 8.8</td>
<td>45.8 ± 10.6^c</td>
<td>49.9 ± 7.7</td>
</tr>
<tr>
<td>PND56</td>
<td>285 ± 30</td>
<td>258 ± 20^c</td>
<td>271 ± 27</td>
</tr>
<tr>
<td>PND70</td>
<td>338 ± 30</td>
<td>323 ± 25</td>
<td>328 ± 27</td>
</tr>
<tr>
<td>PND91</td>
<td>392 ± 21</td>
<td>377 ± 36</td>
<td>386 ± 37</td>
</tr>
<tr>
<td>PND98</td>
<td>424 ± 28</td>
<td>402 ± 41</td>
<td>411 ± 49</td>
</tr>
<tr>
<td>Tissue weight (%)a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>0.85 ± 0.07</td>
<td>0.83 ± 0.07</td>
<td>0.74 ± 0.13^c</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>0.29 ± 0.05</td>
<td>0.28 ± 0.05</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Ventral prostate</td>
<td>0.33 ± 0.06</td>
<td>0.29 ± 0.05^c</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>AGD (%)b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Female pups (N=13) (N=17) (N=15)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Tissue weight (%)$^a$</th>
<th>UVD (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PND56</td>
<td>PND70</td>
<td>PND91</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.15 ± 0.01</td>
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<tr>
<td></td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>PND21</td>
<td>50.0 ± 8.3</td>
<td>42.6 ± 3.6$^c$</td>
<td>51.2 ± 6.8</td>
</tr>
<tr>
<td>PND49</td>
<td>171 ± 20</td>
<td>157 ± 12$^c$</td>
<td>170 ± 13</td>
</tr>
<tr>
<td>PND70</td>
<td>225 ± 19</td>
<td>214 ± 15</td>
<td>219 ± 17</td>
</tr>
<tr>
<td>PND91</td>
<td>251 ± 22</td>
<td>241 ± 18</td>
<td>251 ± 19</td>
</tr>
<tr>
<td>PND98</td>
<td>265 ± 20</td>
<td>250 ± 22</td>
<td>265 ± 20</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.04 ± 0.00</td>
<td>0.03 ± 0.00$^c$</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>AGD (%)$^b$</td>
<td>PND49</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.10 ± 0.00$^c$</td>
<td>0.11 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
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<tr>
<td></td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>UVD (%)$^b$</td>
<td>PND49</td>
<td>0.03 ± 0.00</td>
<td>0.02 ± 0.00$^c$</td>
</tr>
<tr>
<td></td>
<td>0.02 ± 0.00$^c$</td>
<td>0.02 ± 0.00$^c$</td>
<td></td>
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<tr>
<td></td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00$^c$</td>
<td>0.02 ± 0.00</td>
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</table>
a The tissue weight at PND98 is shown as the value relative to the body weight (gram-to-gram percentage).

b AGD and UVD are shown as the value relative to the body weight (millimeter-to-gram percentage).

c Significantly different from control (P < 0.05).

Abbreviations used: AGD, anogenital distance; UVD, urethrovaginal distance; PND, postnatal day.
Fig. 1
Fig. 2
Fig. 3

mRNA level (Control = 1.0)

Hormone level (Control = 1.0)
Fig. 4

**A**

- **LHβ:** Control: 1.2, TCDD: 1.0
- **FSHβ:** Control: 1.4, TCDD: 1.2

**NS**

**B**

- **StAR:** Control: 1.0, TCDD: 5.0
- **CYP1A1:** Control: 0.5, TCDD: 6.0
- **LHβ:** Control: 1.0, TCDD: 1.0
- **FSHβ:** Control: 1.0, TCDD: 1.0

**NS**

*Indicates significant difference.
Fig. 5