Maternal Exposure to Dioxin Imprints Sexual Immaturity of the Pups Through Fixing the Status of the Reduced Expression of Hypothalamic Gonadotropin-Releasing Hormone

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Running title: Dioxin imprints reduction in gonadotropin-releasing hormone

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The number of text pages: 33
The number of tables: 1 (as a Supplemental Table)
The number of figures: 9 (including three Supplemental figures)
The number of references: 49
The number of words in the Abstract: 250
The number of words in the Introduction: 741
The number of words in the Discussion: 1,493

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; eCG, equine chorionic gonadotropin; GD, gestational day; GnRH, gonadotropin-releasing hormone; αGSU, glycoprotein hormone α-subunit; HDAC, histone deacetylase; LH, luteinizing hormone; LV, lateral ventricular; PND, postnatal day; StAR, steroidogenic acute-regulatory protein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic responsive element.
ABSTRACT

Our previous studies have shown that treatment of pregnant rats with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 1 μg/kg) at gestational day (GD)15 reduces the pituitary synthesis of luteinizing hormone (LH) during the late fetal and early postnatal period, leading to the imprinting of defects in sexual behaviors at adulthood. However, it remains unclear how the attenuation of pituitary LH is linked to sexual immaturity. To address this issue, we performed a DNA microarray analysis to identify the gene(s) responsible for dioxin-induced sexual immaturity, on the pituitary and hypothalamus of male pups, at the age of postnatal day (PND)70, born of TCDD-treated dams. Among the reduced genes, we focused on gonadotropin-releasing hormone (GnRH) in the hypothalamus, because of published evidence of role in sexual behaviors. An attenuation by TCDD of GnRH expression emerged at PND4, and there was no subsequent return to the control level. Neither a change in DNA methylation nor histone acetylation accounted for the reduced expression of GnRH. Intracerebroventricular infusion of GnRH to the TCDD-exposed pups after reaching maturity restored the impairment of sexual behaviors. Supplying equine chorionic gonadotropin, an LH-mimicking hormone, to the TCDD-exposed fetuses at GD15 resulted in a recovery from the reduced expression of GnRH as well as the defects in sexual behavior. These results strongly suggest that maternal exposure to TCDD fixes the status of the lowered expression of GnRH in the offspring by reducing the LH-assisted steroidogenesis at the perinatal stage, and this is the mechanism for the imprinting of defects in sexual behaviors at adulthood.
Introduction

2,3,7,8-Tetrachlorodibenzo-\(p\)-dioxin (TCDD) is the most toxic dioxin known, and its adverse effects on humans and wildlife through the food chain are a major cause for concern (Larsen, 2006; Schecter et al., 2006). In laboratory animals, maternal exposure to TCDD causes a number of disorders in the offspring, such as sexual immaturity and growth retardation (Peterson et al., 1993). For example, in the pups born from dams exposed to TCDD, severe defects after growing up have been observed in spermatogenesis (Mably et al., 1992; Gray et al., 1995; Faqi et al., 1998) and gender-specific sexual behaviors (Mably et al., 1992; Bjerke and Peterson, 1994; Gray et al., 1995; Faqi et al., 1998). These disorders seem to be serious because they are caused by TCDD at much lower doses than those needed for other forms of toxicity (Poland and Knutson, 1982). Although many studies have been conducted to clarify the mechanism underlying the above toxicity in the pups, much more information is needed to give us a better understanding.

Our previous studies have demonstrated that maternal treatment with TCDD at the late gestational stage attenuates the pituitary production of luteinizing hormone (LH), a primary regulator of gonadal steroidogenesis, during the perinatal stage (Mutoh et al., 2006; Taketoh et al., 2007; Takeda et al., 2009, 2011, 2012; Koga et al., 2012). More specifically, treating pregnant Wistar rats at gestational day (GD)15 with 1 \(\mu\)g/kg TCDD transiently reduces the expression of LH in both pituitary mRNA and circulating hormone levels during a period from GD20 to postnatal day (PND)0 (Takeda et al., 2012). In accordance with this, TCDD reduces the testicular expression of steroidogenic proteins, including steroidogenic acute-regulatory protein (StAR), a
cholesterol-transporting protein associated with a rate-limiting process of steroidogenesis (Manna et al., 2009), and cytochrome P450 (CYP) 17, an essential enzyme for sex-steroid biosynthesis (Payne and Youngblood, 1995), in late fetuses and early neonates (Mutoh et al., 2006; Takekoh et al., 2007; Takeda et al., 2009, 2012). In one of the above studies, direct supplementation of equine chorionic gonadotropin (eCG), an LH-mimicking hormone, into the fetuses exposed to TCDD at GD15 was shown to restore not only the attenuated expression of fetal steroidogenic proteins but also the impairment of sexual behaviors after reaching maturity (Takeda et al., 2009). These observations strongly suggest that TCDD initially reduces LH biosynthesis in the fetal and neonatal pituitary, resulting in the imprinting of defects in sexual behaviors at adulthood. However, the mechanism whereby the transient suppression of the LH-stimulated syntheses of sex steroids during the fetal and neonatal stage imprints sexual immaturity after maturation remains largely unknown.

It has long been believed that sex steroids are essential for the brain and sexual differentiation that occurs in a short window of the period around birth, the so-called ‘critical period’ (Whalen and Edwards, 1967; MacLusky and Naftolin, 1981; Morris et al., 2004; McCarthy, 2008). In this period, the transient stimulation of the hypothalamus by sex steroids is likely to trigger a permanent change in neural structure and cellular metabolome including hormones and neurotransmitters so as to acquire gender-specific phenotypes (Morris et al., 2004; McCarthy, 2008). Very confusingly, brain and sex differentiation must be concerted with various mechanisms in a target gene-, brain region- and exposing timing-specific manner (Davies and Wilkinson, 2006; McCarthy, 2008). Because maternal exposure to TCDD damages testicular steroidogenesis during the critical period (Mutoh et al., 2006; Takekoh et al., 2007; Takeda et al., 2009, 2012), it
is conceivable that TCDD disturbs the normal development of the hypothalamus to imprint the abnormality of gene expression and/or neural structure, leading to the impairment of sexual maturation such as the acquisition of sexual behavior.

To address the above issue, we initially performed DNA microarray analysis to identify gene(s) whose expression was associated with defects in sexual behaviors. Based on the data obtained, we focused on the lowered expression of gonadotropin-releasing hormone (GnRH). This is a decapeptide hormone secreted from hypothalamic neurons, and plays a pivotal role in the maintenance and development of reproductive function by stimulating gonadotropin/sex-steroid biosyntheses (McCann, 1977). In addition, GnRH receptors are abundantly expressed in some regions of the brain (Wen et al., 2011), and their contribution to the gender-specific sexual behaviors has been demonstrated (Dorsa and Smith, 1980; Sirinathsingji, 1983; Meredith and Howard, 1992). We then investigated whether a TCDD-produced reduction in hypothalamic GnRH is directly linked to defects in sexual behaviors at adulthood and the mechanism for the fixation of the lowered expression of GnRH.

Materials and Methods

Materials. TCDD was obtained from AccuStandard, Inc. (New Haven, CT). All other reagents were of the highest grade commercially available.

Animals. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kyushu University. Female (7 weeks-old) and male (10
weeks-old) Wistar rats were purchased from Kyudo Co. Ltd. (Tosu, Japan). All animals were fed a standard chow (CE-2; CLEA Japan, Tokyo, Japan) and sterilized water ad libitum, and they were kept in an environmentally-controlled room maintained at 22 ± 5 °C and 50 ± 15% relative humidity under a 24 hr light/dark cycle (light period, 7:00 AM-7:00 PM). Female rats were paired overnight with male rats. Next morning, the presence of sperm in the vaginal smears was checked by microscopy (×400) to confirm pregnancy. When sperm was detected, the day was designated as GD0 of pregnancy, and the pregnant rats were isolated for the subsequent experiment. Pregnant rats at GD15 were given an oral dose of TCDD (1 μg/kg/2 ml corn oil) or corn oil alone. Then, tissues and blood were collected from the fetuses and born pups at GD18, 19, 20 and 21, and PND0, 2, 4, 7, 10, 14, 28, 56 and 70 (PND0 means the birth day).

Treatment of Fetuses. Direct injection of eCG into fetuses was conducted according to the method reported previously (Mutoh et al., 2006). Briefly, GD17 pregnant dams pre-exposed to TCDD (1 μg/kg, p.o., GD15) were anesthetized by inhalation of 5% sevoflurane, and the anesthesia was maintained with 2% sevoflurane during operation. The uterus was pulled out from the dam, and eCG (Sigma-Aldrich Co., St. Louis, MO; 5 I.U./5 μl saline) or vehicle alone was injected into the backs of the fetuses. After treatment, the uterus containing fetuses was returned to the abdomen, and the surgical wound was sewn up. The tissues of fetuses (GD20) and born pups (PND30) from operated dams were used for RT-PCR and ELISA analysis, and the sexual behavior was examined using male pups at PND70.

Intracerebroventricular Infusion. Intracerebroventricular supplementation of
GnRH to male pups was performed one week before the first test of sexual behavior. This involved the male pups at the age of PND63 (9 weeks-old) which were born from the dams treated with TCDD or vehicle at GD15 being anesthetized by an intraperitoneal injection of a mixture (10 ml saline/kg) consisting of medetomidine (0.15 mg/kg), midazolam (2 mg/kg) and butorphanol tartrate (2.5 mg/kg), and their heads then being fixed on a stereotaxic instrument (IMPACT-1000B; Muromachi kikai Co., Ltd., Tokyo, Japan). A 15-mm incision was made in the head skin, and an infusion cannula connected to a catheter tube (brain infusion kit 2: DURECT Corp., Cupertino, CA) was inserted into the lateral ventricle (LV) to a depth of 5 mm and at the position described below: antero-posterior, -1.0 mm; and lateral, 1.0 mm from the bregma. The other end of the catheter tube was connected to an osmotic mini-pump (model 2002, DURECT) filled with 200 μl GnRH (200 ng/μl; Peptide Institute, Inc., Osaka, Japan) which was dissolved in sterilized saline containing 0.1 M HCl. The pump was subcutaneously embedded in the back of the operated rats. The surgical wound in the head was then stitched up, and the capacity for sexual behavior was examined one and two weeks after the operation. The correct distribution of the injected solution to the LV and its diffusion into other ventricles were checked by infusing a 0.1% aqueous solution of bromophenol blue. The control rats were similarly operated on and fitted with a cannula, through which vehicle not containing GnRH was infused.

**Sexual Behavior.** Sexual behavior in male rats was examined at the age of 10 and 11 weeks of age according to the method described elsewhere (Takeda et al., 2009). Each test rat was transferred to a testing cage in the morning, and paired with a rutted female (8-10 weeks-old), a sex partner, in the night (20:00-24:00). Rutted females were
prepared in advance by ovariectomy and subcutaneous injection of 17β-estradiol (50 μg) and progesterone (1 mg) 54 and 6 hr prior to the test, respectively. The capacity for sexual behavior was evaluated using the number of mounts and intromission behavior for 30 min, and the time until the first mount and intromission.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** The expression of mRNAs was quantified by real-time RT-PCR according to the method described previously (Matsumoto et al., 2010). In brief, total RNA was extracted from the testis, pituitary, hypothalamus, cerebrum and cerebellum using RNeasy Kits (QIAGEN GmbH, Hilden, Germany). The RNA (100-250 ng) obtained was treated with gEraser (TaKaRa-bio, Shiga, Japan) to digest contaminating genomic DNA, and reverse-transcribed to its cDNA. Target mRNAs were amplified with Fast SYBR Green Master Mix (Invitrogen, Carlsbad, CA), using a StepOnePlus Real-time PCR system (Invitrogen). The primer designs are shown in the Supplemental Table 1. The PCR conditions were as follows: 95°C, 20 sec-40 cycles (95°C, 3 sec-60°C, 30 sec). The amount of quantified target mRNA was normalized by β-actin mRNA.

**Enzyme-linked Immunosorbent Assay (ELISA).** The concentration of hypothalamic, cerebral and cerebellar GnRH was quantified using an ELISA kit (Uscn Life Sciences Inc., Houston, TX). The tissues were homogenized with 9-volumes relative to the tissue weight of saline containing 0.1 M HCl, and centrifuged at 5,000×g for 5 min. Before assay, the supernatants from the cerebellum and hypothalamus were diluted 2-times with H2O. The supernatant from the cerebrum was used without dilution.
DNA microarray. This was conducted according to the method reported previously (Takeda et al., 2012). Briefly, total RNA was isolated from the pituitary and hypothalamus of PND70 male pups born from the dams treated with TCDD (1 μg/kg) or vehicle at GD15, using an RNeasy Mini Kit (QIAGEN). The total RNA (250 ng) was converted to its biotinylated-cRNA according to the manufacturer’s instructions (Illumina TotalPrep RNA Amplification Kit; Ambion, Austin, TX). A total of 1500 ng biotinylated-cRNA was overlaid onto individual array spots of the rat microarray chip (RatRef12-v1 BeadChip; Illumina). The data on gene expression were compiled by Bead Studio software (Illumina). The microarray datasets were submitted to the GEO database (accession number: GSE48100; www.ncbi.nlm.nih.gov/geo/ provided in the public domain by NCBI). The signal intensity for gene expression was normalized using a quantile algorithm. To achieve this, the ‘preprocessCore’ library package (Bolstad et al., 2003) in Bioconductor software (Gentleman et al., 2004) was used. The genes agreeing with the criterion that their expression was detected in 4 or more samples out of a total of 6 samples (3 controls + 3 TCDD-treated samples) at detection p-values of more than 0.05 were selected and further analyzed. To identify pairs showing a significant difference, the data were then processed using a package in the Bioconductor, Linear Models for Microarray Analysis (limma) (Smyth, 2005), and a significant difference was identified using a criterion set at limma $P < 0.05$.

Analysis of DNA Methylation. The methylation status of the GnRH gene was analyzed by bisulfite DNA sequencing analysis (Frommer et al., 1992) according to the procedures reported previously (Takeda et al., 2012). The genomic DNA extracted from
the hypothalamus of male rats at PND14 and PND30 was treated with sodium bisulfite, using an EpiTect Plus DNA Bisulfite Kit (QIAGEN). The modified DNA was amplified by EpiTaq™ HS (Takara-bio) with the following primers: GnRH_site 1 (forward), 5'-AAGAGTAGGGAAGAAGTTGTG-3', GnRH_site 1 (reverse), 5'-CCTTCCCTTTAAAAATCTCTCC-3', GnRH site 2 (forward), 5'-GTGGATTATAGAGTAAAGATAG-3', and GnRH site 2 (reverse), 5'-AACTTTTAAACCCTCACAAAAAACCA-3'. The conditions used for PCR amplification were: 95°C for 3 min-40 cycles (95°C for 30 sec-56°C for 30 sec-72°C for 1 min)-72°C for 5 min. Each DNA fragment was purified using a QIAquick gel extraction kit (QIAGEN), inserted by TA ligation into a nicked pGEM-T Vector (Promega, Madison, WI), and this was transfected to JM109 competent cells for cloning. The plasmids were prepared from transformant colonies, and the sequences of their passenger DNAs were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen) and the primer, 5'-TCAAGCTATGCATCCAACGC-3'. The plasmids obtained from 10 transformant colonies/one pup sample underwent sequencing.

Statistical Analysis. The data for the pups in one dam were averaged to become a single analytical unit. The statistical difference between the control and TCDD group was evaluated by Student's t-test. The comparison among multiple groups was conducted by one-way analysis of variance with a post-hoc test (Turkey's multiple comparison test), using GraphPad Prism Version 5 software (GraphPad Software, Inc., SanDiego, CA). The statistical significance was set at p<0.05.
Results

TCDD-Produced Defects in Sexual Behavior Due to Damage to the Pituitary-Gonad Axis during the Perinatal Period. Firstly, we analyzed the expression of steroidogenic proteins and their regulatory hormones in the testis and pituitary from GD19 to PND7 to re-confirm the age-specificity of TCDD-damage to the pituitary-gonad axis. When pregnant rats were given an oral dose of TCDD (1 μg/kg) at GD15, the expression of testicular mRNAs coding for StAR, CYP17, CYP11A1 and 3β-hydroxysteroid dehydrogenase (HSD) was attenuated during a period from the late fetal (GD20 and 21) to early neonatal (PND2) stages (Fig. 1A and Supplemental Fig. 1). In accordance with changes in StAR/CYP17, maternal exposure to TCDD attenuated the fetal and neonatal expression of pituitary LH in a β-subunit-specific manner (Fig. 1B). The direct supplementation of eCG, an LH-mimicking hormone, to the GD17 fetuses pre-exposed to TCDD at GD15 completely restored a TCDD-induced attenuation in not only StAR/CYP17 mRNAs in the fetal testis (Fig. 1C), but also the frequency of mount, one of the masculine sexual behaviors, in male pups after reaching maturity (Fig. 1D). These observations agree with our previous demonstration that maternal exposure to TCDD disrupts testicular steroidogenesis in the late fetal and early neonatal period by targeting pituitary LHβ expression, leading to the imprinting of defects in sexual behaviors at adulthood.

DNA Microarray Analysis to Identify the Target Gene Linked to Defects in Sexual Behaviors. To identify the genes which are linked to the imprinting of sexual
immaturity by TCDD, we performed a DNA microarray analysis, using the pituitary and hypothalamus prepared from PND70 male pups which were born from dams treated with TCDD at GD15. The result obtained showed that many changes in gene expression remain in both tissues even although the timing of exposure to TCDD is at the fetal stage. In the hypothalamus, 332 and 283 genes were significantly up- and down-regulated by TCDD, respectively (Fig. 2A: refer to GSE48100 registered to NCBI database for details of the altered genes). Also in the pituitary, 281 and 323 genes increased and decreased, respectively (Fig. 2A). While we were unable to pinpoint any linkage between altered genes in the pituitary and defects in sexual behaviors, we found that TCDD reduces the hypothalamic expression of GnRH, a regulator of sexual behavior (Fig. 2A). Further analysis by real-time RT-PCR revealed that the reduced level of GnRH mRNA is never restored to its original level (PND4) even at PND70 (Fig. 2B). An attenuation in GnRH level was also observed in the hormonal level in the cerebrum and cerebellum as well as the hypothalamus (Fig. 2C). However, TCDD lacked the ability to reduce the cerebral and cerebellar expression of GnRH mRNA in the male pups at PND70 (Fig. 2D). Thus, a reduction in GnRH content in the brain tissues appears to originate from the reduced synthesis in the hypothalamus. These results suggested that the lowered expression of GnRH in the neonatal brain is the major mechanism for the imprinting of TCDD-produced sexual immaturity at adulthood.

**Recovery from the TCDD-Produced Sexual Immaturity by Direct Supplementation of GnRH to Grown-up Pups.** We then investigated whether the lowered expression of GnRH contributes to TCDD-induced sexual immaturity. For this, GnRH was intracerebroventricularly infused using an osmotic pump to male pups born
from TCDD-treated dams, and their activity of masculine copulatory behavior was examined at PND70. The pups which were born from TCDD-exposed dams and had grown-up exhibited prolonged latency in mount and intromission, and reduced frequencies of such behaviors (Fig. 3). The intracerebroventricular supplementation of GnRH to grown-up pups from TCDD-treated dams restored all those defects (Fig. 3). These results strongly suggest that fixing the status of the lowered expression of GnRH plays a crucial role in the sexual immaturity produced by maternal exposure to TCDD.

Absence of a TCDD Effect on the Epigenetic Regulation of GnRH Gene. To examine the mechanism underlying TCDD-induced GnRH reduction, we investigated whether TCDD affects the status of methylation at the site 5'-upstream of the GnRH gene, using the bisulfite DNA sequencing method. A previous study has suggested that a change in GnRH gene expression during development is produced by altering the methylation status of the 5'-upstream region (Kurian et al., 2010). However, when we analyzed CpG-rich sites found in the two 5'-upstream regions of the GnRH gene at PND14 and 30, TCDD did not alter the methylation status at these sites (Fig. 4). Quite recently, this laboratory found that TCDD induces the pituitary expression of histone deacetylases (HDACs) during the fetal and neonatal period to reduce the expression of LHβ (Takeda et al., 2012). However, TCDD hardly affected the expressed level of HDACs in the hypothalamus of PND14 pups (Supplemental Fig. 2). These data suggest that TCDD fixes the status of the lowered expression of brain GnRH in male offspring by a mechanism distinct from the influence on DNA methylation and histone acetylation.
Recovery from a TCDD-Induced Attenuation in GnRH expression by eCG Treatment. It is conceivable that TCDD impairs the maturation of GnRH neurons via damage to perinatal steroidogenesis, resulting in a permanent reduction in GnRH supply. To address this hypothesis, TCDD-exposed fetuses were directly treated with eCG, an upstream regulator for steroidogenesis, and the effect of this treatment on the expression of brain GnRH was examined. When the hypothalami of pups that had reached PND30 were examined, a TCDD-induced reduction in GnRH was restored by treating GD17 fetuses with eCG (Fig. 5A). A recovery produced by eCG supplementation was also observed in the hormonal level of GnRH in the hypothalamus, cerebrum and cerebellum at 11 weeks of age (Fig. 5B). These observations strongly support the view that TCDD fixes the status of the lowered expression of brain GnRH by attenuating perinatal steroidogenesis.

Discussion

As shown previously and re-confirmed by this study, TCDD damages the production of pituitary LH in a perinatal age- and β-subunit-specific fashion and, as a consequence of this change, the phenotype of impaired sexual behaviors is imprinted (Mutoh et al., 2006; Taketoh et al., 2007; Takeda et al., 2009, 2012). However, until now, it remained largely unknown how a reduction in sex-steroid level produced by lowering gonadotropin expression was linked to abnormal sexual behaviors. The present study has provided evidence that fixing the status of the lowered expression of GnRH in male pups renders them sexually immature after reaching maturity. While GnRH is
regarded as a main regulator of reproductive function (McCann, 1977), this peptide also promotes the expression of gender-specific sexual behaviors (Dorsa and Smith, 1980; Sirinathsinghji, 1983; Meredith and Howard, 1992). The latter role of GnRH is based on the following pieces of evidence: 1) intracerebroventricular injection of GnRH facilitates mounting behavior (Dorsa and Smith, 1980); and 2) GnRH produces recovery from a defect in the intromission activity of the male rats which was lost by operation on the vomeronasal organ, a region contributing to reproductive function (Meredith and Howard, 1992). In this study, we observed that TCDD-impaired sexual behaviors were restored to normal by compensating for a shortage of brain GnRH with the exogenous hormone. This is compelling evidence supporting the crucial role of GnRH in sexual behaviors. In addition, direct supplementation of eCG, an LH-like hormone, to fetuses blocked all TCDD effects on 1) gonadal steroidogenesis in fetuses, 2) GnRH expression by the postnatal male pups, and 3) masculine sexual behavior at adulthood. These results strongly suggest that maternal exposure to TCDD targets the pituitary expression of LH during the perinatal stage to attenuate testicular steroidogenesis, leading to a continuous regression in the expression of brain GnRH, which is a determinant for sexual maturation (Fig. 6).

In this study, male rats were intracerebroventriculare replenished with GnRH at an infusion rate of 100 ng/hr, using an osmotic pump. This experimental condition was selected for the following reasons and based on information: 1) an intracerebroventricular injection of 50 ng/30 min GnRH restores defects in sexual behaviors induced by removal of the vomeronasal organ (Meredith and Howard, 1992); 2) GnRH exhibits only a brief duration of effectiveness because of its short half-life under in vivo conditions (Sirinathsinghji, 1983; Lasdun et al., 1989), and 3) probably,
the effect of GnRH given by injection easily varies depending on the experimental conditions, such as the timing of surgery and injection, and the age of the animals used (Dorsa and Smith, 1980). Although it is of concern that the GnRH contained in an osmotic pump implanted in rats is degraded during the test period (2 weeks), we confirmed in the preliminary study that even after incubating aqueous GnRH solution at 37°C for 2 weeks, about the half the GnRH remains in unchanged form (data not shown).

TCDD did not affect the expression of cerebral and cerebellar GnRH mRNA, whereas the hormonal level of GnRH in both tissues was attenuated by TCDD treatment. A reduction by TCDD in the expression of GnRH mRNA was only seen in the hypothalamus. Some kinds of neurons in the hypothalamus-pituitary axis terminate at the median eminence region, and this area is suggested to be a major source of supply of GnRH to the cerebroventricular system (Caraty and Skinner, 2008). Clements et al. (2009) have demonstrated that treatment of pregnant rats with 5 μg/kg TCDD markedly impairs the function of the GnRH secretory system in their pups after reaching maturity. Taken together, a TCDD-induced reduction in brain GnRH seems to occur through initial damage to hypothalamic gene regulation. Alternatively, TCDD may attenuate not only the hypothalamic synthesis of GnRH but also its function secreting GnRH to the cerebroventricular regions. In agreement with this hypothesis, supplying eCG to fetuses restored the cerebral and cerebellar concentration of GnRH protein as well as the hypothalamic expression of GnRH.

It is well known that testosterone and 17β-estradiol activate masculine sexual behaviors by binding to their receptors (Ogawa et al., 2000; Sato et al., 2004). From these observations, there is another possibility that a TCDD-induced reduction in
hypothalamic GnRH disrupts gonadal steroidogenesis through damage to LH biosynthesis, resulting in the emergence of impaired sexual behaviors. In our microarray data, however, neither the expression of LHβ nor αGSU mRNA in the PND70 pituitary was attenuated by in utero exposure to TCDD. In accordance with this, treating pregnant rats with TCDD does not show any effect on the pituitary LH level and/or gonadal steroidogenesis of the pups after reaching maturity (Mably et al., 1992; Gray et al., 1995; Faqi et al., 1998). It should be noted that one report has claimed a TCDD-dependent decrease in the level of plasma testosterone in Holtzman rats (Bjerke and Peterson, 1994). Therefore, it appears likely that the effect of GnRH supplementation on the TCDD-induced defects in sexual behaviors occurs through direct action on the brain rather than stimulation of sex-steroid biosyntheses.

Besides the role as the stimulator of sexual behavior, GnRH is a well-known factor regulating the pituitary synthesis of gonadotropins such as LH (McCann, 1977). Therefore, it seems strange that LHβ returns to the normal level in the pups born of TCDD-treated dams, even although GnRH expression continues to be suppressed after birth. In this context, the regulation of LH biosynthesis by GnRH is very complicated, and GnRH must be secreted to stimulate LHβ transcription in a pulsatile manner (Burger et al., 2004). A change in GnRH pulsatile pattern leads to the reduced expression of LH (Burger et al., 2004; Oakley et al., 2009). However, the total amount of GnRH seems not to be a major factor regulating LH biosynthesis, because circulating LH maintains its normal concentration, even if the GnRH input falls to below 20% of the normal level (Mayer and Boehm, 2011). Taken together, it is reasonable to consider that TCDD reduces the hypothalamic expression of GnRH and its supply to the brain without affecting GnRH pulsatile secretion.
The mechanism whereby a shortage of perinatal sex-steroids defines the reduced expression of GnRH in the developmental process remains unclear. This study has revealed that the reduced level of GnRH mRNA never returns from its onset level at PND4, which is the day involved in a window of the ‘critical period’ for brain organization/differentiation including neuronal maturation (Whalen and Edwards, 1967; MacLusky and Naftolin, 1981). This finding proposes one possible mechanism that reducing perinatal steroidogenesis impairs the maturation of GnRH neurons in the offspring to produce a permanent reduction in GnRH expression (Fig. 6). In this context, it has been suggested that epigenetic mechanisms, such as DNA methylation and histone acetylation, regulate the transcription of the GnRH gene during the period when neuronal maturation takes place (Kurian et al., 2010; Iyer et al., 2011). However, in the present study, little change in DNA methylation of the GnRH gene and HDAC expression was observed in the hypothalamus of TCDD-exposed pups. Hence, the reason why TCDD reduces the hypothalamic expression of GnRH does not seem to be explained by a change in epigenetic regulation. A number of GnRH neurons gather in the hypothalamic area including the sexually-dimorphic nucleus-preoptic area (SDN-POA), a central region serving to produce sexual differentiation (Avigdor et al., 2005; Wray, 2010). Maternal exposure to a low dose of TCDD reduces the volume of SDN-POA in male pups (Ikeda et al., 2005). On the other hand, perinatal exposure to TCDD is suggested to reduce the viability of neural cells by increasing apoptosis (Tomasini et al., 2012). However, many more studies focusing on maturation and neuron dropout will be needed to gain a better understanding about the mechanism governing a TCDD-produced reduction in hypothalamic GnRH mRNA.

In general, a number of toxic effects produced by TCDD are believed to occur
following the activation of a nuclear receptor, the aryl hydrocarbon receptor (AhR) (Poland and Knutson, 1982; Fernandez-Salguero et al., 1996; Mimura et al., 1997). The AhR-dioxin complex binds to its cognate sequence, the xenobiotic responsive element (XRE), present in the 5’-upstream region of the target genes to produce a change in gene expression (Fujisawa-Sehara et al., 1987; Mimura and Fujii-Kuriyama, 2003). A computer-assisted survey of the GnRH gene sequence suggested the presence of a core XRE motif at -3,786 bp of the 5’-upstream regions. However, while maternal exposure to TCDD at GD15 dramatically induced the hypothalamic expression of CYP1A1, an AhR-responsive gene, in the late fetuses and early infants, this induction disappeared in the pups at PND56 and 70 (Supplemental Fig. 3). Thus, it is unlikely that the AhR-TCDD complex directly reduces the hypothalamic expression of GnRH in the pups after reaching maturity. To clarify the role of AhR more definitively, generation of AhR-null rats is now underway in this laboratory. Further studies are also needed focusing on the maturation and/or degeneration of GnRH neurons for deeper understanding the mechanism whereby maternal exposure to TCDD fixes the status of lowered expression of GnRH.
Acknowledgements: The authors thank Michi Amago, Takanori Nakamura and the other staff of the Research Support Center (Graduate School of Medical Sciences, Kyushu University) for technical support during the DNA microarray and sequencing analysis.

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Participated in research design: Takeda, Ishii, Himeno, and Yamada
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Contributed new reagents or analytic tools: Yamamoto, and Shimazoe
Performed data analysis: Takeda, Fujii, and Hattori
Wrote or contributed to the writing of the manuscript: Takeda, and Yamada
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This work was supported by grants from the Japan Society for the Promotion of Science [Grant-in-Aid for Young Scientist (B) 23790152 and Scientific Research (S) 24221004], and the Ministry of Health, Labour and Welfare, Japan [Research on Food Safety (H24-Designated Research-014)].

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Fig. 1. The effect of maternal exposure to TCDD on the expression of steroidogenic proteins and LH during the perinatal stage, and recovery from the TCDD-produced defects in sexual behaviors at adulthood by in utero injection of eCG. In experiments A and B, pregnant dams at GD15 were exposed to TCDD (1 μg/kg, p.o.), and the testis (A) and pituitary (B) of fetuses/neonates were removed during GD19 and PND7. The mRNAs coding for testicular StAR and CYP17 (A), and pituitary αGSU and LHβ (B) were determined by real-time RT-PCR, and normalized by β-actin mRNA. Each value represents the mean ± S.E.M. of 6-10 fetuses (neonates) which were removed (born) from different dams. Significantly different from the control group; *p<0.05, **p<0.01 and ***p<0.001. The inset in panel B is a graph which magnifies the data of GD19-21.

In C and D, the fetuses exposed to TCDD (maternal dose of 1 μg/kg, GD15) were treated directly with eCG (5 I.U.) at GD17. The testicular expression of StAR and CYP17 mRNAs at GD20 (C) and the capacity of masculine sexual behavior after reaching maturity (D) were measured. Bars are the means ± S.E.M. of 5-9 rats. Significantly different between the pair indicated; *p<0.05 and **p<0.01.

Fig. 2. A reduction in the expression of hypothalamic GnRH in male rats born from dams exposed to TCDD. A, microarray analysis of hypothalamic and pituitary mRNAs in male adult rats (PND70) born from dams exposed to TCDD (1 μg/kg, GD15). The heat map was generated by MeV software (Dana-Farber Cancer Institute, Boston, MA). Genes whose expression is significantly increased and decreased by maternal exposure to TCDD (1 μg/kg) are shown in red and green, respectively (p<0.05).
magnitude of the alteration, see color gradation shown beneath the figure. Respective lanes are different pups which were born from 3 different dams. Several genes showing a significant change after TCDD treatment are indicated. B, GnRH mRNA levels on GD18 and PND70 were determined by real-time RT-PCR, and normalized by β-actin mRNA. Each plot represents the mean ± S.E.M. of 6-10 fetuses (pups) which were removed (born) from different dams. C, the hormonal level of GnRH in the hypothalamus, cerebrum and cerebellum was measured by ELISA. Each bar represents the mean ± S.E.M. of 4-7 pups which were born from different dams. D, the expression of GnRH mRNA at PND70 was determined by real-time RT-PCR, and normalized by β-actin mRNA. Each bar represents the mean ± S.E.M. of 4-6 pups which were born from different dams. Abbreviations used: NQO1, NAD(P)H quinone oxidoreductase 1.

**Fig. 3.** Recovery by intracerebroventricular infusion of GnRH from the TCDD-induced defects in sexual behaviors of male rats. Male pups at 9 weeks born from dams treated with TCDD at GD15 underwent intracerebroventricular infusion using a cannula-connected osmotic pump filled with 200 ng GnRH/μL saline containing 0.1 M HCl. The operated rats were continuously infused with GnRH at a rate of 100 ng/hr throughout the experiment (for 2 weeks). The latency until the first mount (A), the number of mounts (B), the latency until first intromission (C) and the number of intromissions (D) were observed at the age of 10 and 11 weeks after pairing with receptive females (pairing ratio, male : female = 1 : 1). Bars are the means ± S.E.M. of 14 rats. Significant differences between pairs indicated: *p<0.05, **p<0.01 and ***p<0.001.
Fig. 4. Absence of the TCDD effect on the DNA methylation of the GnRH gene. A, bisulfite DNA sequencing was performed to analyze the methylation status of GnRH genes. In short, genomic DNA was extracted from the male hypothalamus, treated with sodium bisulfite, and then the 5′-upstream regions of GnRH genes were amplified by PCR. The amplified DNA was inserted into a plasmid vector, transfected to JM109 competent cells for cloning, and the cloned plasmid was subjected to sequencing (see the experimental section for the details). Ten colonies/one pup sample were taken, and their plasmids were sequenced. In this experiment, 3 pups at PND14 (pup No. 1-3) and 3 pups at PND30 (pup No. 7-9) were removed from different control dams (one pup/one dam), and another 2 x 3 pups (pup No. 4-6 and No. 10-12) were also obtained from different dams treated with TCDD at GD15. Each row of circles represents a single clone. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. The number shown at the top of panel A indicates the location of CpG. In (B), bars were calculated as the percentage of methylation in each CpG site, and represent the mean ± S.E.M. of 3 pups.

Fig. 5. Restoration of a TCDD-produced attenuation in the expression of GnRH by direct supplementation of eCG to fetuses. Pregnant rats were exposed to TCDD (1 μg/kg, p.o.) at GD15, and the fetuses were injected with eCG at GD17. A, the hypothalamic expression of GnRH mRNA at PND30 was analyzed by real-time RT-PCR, and normalized by β-actin mRNA. B, the GnRH concentration in the hypothalamus, cerebrum and cerebellum at 11 weeks old was measured by ELISA. Each bar represents the mean ± S.E.M. of 6-10 rats born from the different dams. Significant differences between pairs indicated: *p<0.05, **p<0.01 and ***p<0.001.
Fig. 6. Postulated mechanism for the TCDD-mediated imprinting of impaired sexual behavior. Maternal exposure to TCDD disrupts the testicular steroidogenesis during the late fetal and early postnatal stage by reducing the expression of pituitary LH. Such transient defects imprint the status of lowered expression of hypothalamic GnRH, leading to impaired sexual behavior after reaching maturity. The mechanism underlying a GnRH reduction may be a disturbance of GnRH neural maturation through reducing perinatal steroidogenesis.

Abbreviations used in this figure: HT, hypothalamus; PI, pituitary; GnRH-N, GnRH neuron.
Fig. 2

A. Heatmap showing gene expression changes in the Pituitary and Hypothalamus under control and TCDD conditions.

B. Graph showing mRNA level (% of β-actin) over GD and PND, comparing control and TCDD conditions.

C. Bar graphs showing GnRH protein levels (µg/mg tissue) in the Hypothalamus, Cerebrum, and Cerebellum under control and TCDD conditions.

D. Further bar graphs showing GnRH mRNA levels (% of β-actin) in the Cerebrum and Cerebellum under control and TCDD conditions.

* indicates statistical significance.
Fig. 3
Fig. 4

A

B