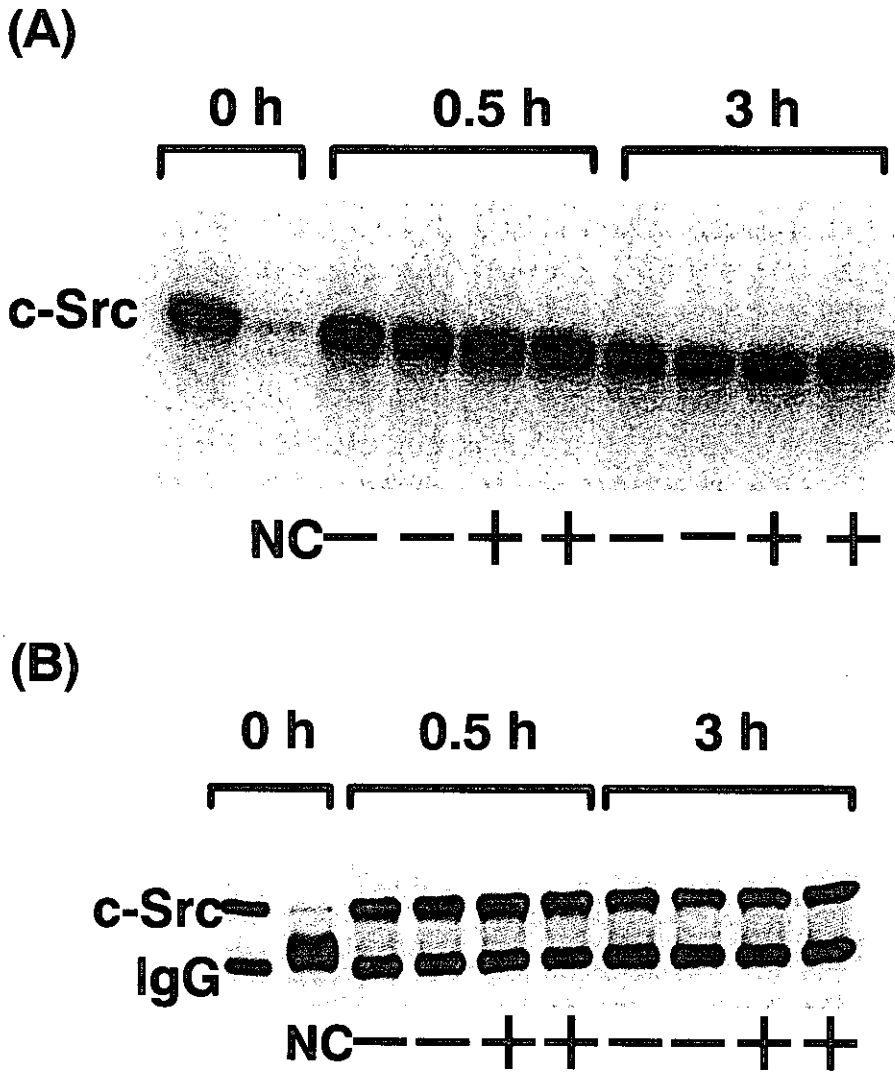
図3



厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

コプラナーPCB と TCDD の学習行動への影響の比較解析

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研究要旨

ダイオキシン類への曝露が発達中の高次脳機能の発達を障害する可能性が示唆されている。本研究では、Long-Evans ラットにおけるオペラント条件づけ行動試験を用い、2,3,7,8 四塩素化ジベンゾパラジオキシン（TCDD）あるいは PCB126 それぞれの発達期曝露が成熟後の記憶・学習機能に及ぼす影響について、比較検討した。母ラットが妊娠 15 日目に 50、200、800ng/kg の TCDD と PCB126 (TEF0.1) を 500、2000、8000 ng/kg（それぞれ 50, 200, 800 ng TEQ/kg)、を経口的に単回曝露した。生まれた仔ラットが成熟後にオペラント行動試験を行った。オペラント行動試験では、2 種類の課題を交互に提示する多元強化スケジュールを 30 日間行った。その結果、TCDD のオペラント行動に対する毒性は曝露用量依存的ではなく、逆 U 字を示す曝露用量特異的な影響としてあらわれた。PCB126 の毒性も同様のパターンを示した。PCB126 の相対毒性強度は、TCDD と同様もしくは若干低めのであった。さらに最高用量曝露群では、PCB126 は TCDD よりも強い毒性も示したことから、PCB126 の「非 TCDD 毒性」の存在も示唆された。

研究協力者
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A. 研究目的

2,3,7,8-*p*-四塩素化ジベンゾパラジオキシン (TCDD)は、発癌性、免疫毒性、生殖毒性など人体に種々の毒性をもつことが懸念されている(Tohyama, 2000)。さらに TCDD は、高次脳機能に影響することも示唆されており、妊娠ラットへの投与が胎仔成長後の学習行動に影響することが報告されている（Hojo, et al., 2002)。PCB に関しても、ヒトにおける疫学調査で母親の血中 PCB 濃度と子供の IQ に高い相関関係があることが報告されているが(Stewart et al. 2000)、根拠となるデータが少なく、PCB の直接影響なのか断定できない状況にある。また、TCDD と PCB の毒性の異同も未だ解明されていない。そこで本研究では、妊娠期の TCDD あるいはコプラナーPCB である 3, 3', 4, 4', 5-五塩素化ビフェニル(PCB126)の母体曝露が発達中の胎子脳に及ぼす影響について、記憶学習行動に焦点を当て検討した。

B. 研究方法

1. 試験材料

TCDD、PCB126 (N ノナン 0.5%)のコーンオイル溶液(N ノナン 0.5%)を、ラット用胃ゾンデで経口投与した。コントロール群にはコーンオイル(N ノナン 0.5%)を同様に曝露した。

2. 実験動物および飼育条件

Long-Evans ラットは、チャールズリバーから購入した。飼育および処理はすべて国立環境研究所の動物実験手順に従い、特殊化学物質管理区域内にて行った。

3.曝露方法

妊娠 15 日目の Long-Evans ラットに、

TCDDを 50, 200, 800 ng/kg (それぞれT50, T200, T800群、各 8, 7, 8,匹)またはPCB126を 500, 2000, 8000 ng/kg (P500, P2000, P8000群、各 8, 8, 9 匹)、コントロール群としてコーン油溶液(8 匹)を単回経口曝露した。その後に生まれた仔動物に対し、生後 80 日目以降実験終了まで給餌制限を行い(1日あたり雄 10g、雌 8g)、体重を雄 290-330g、雌 200-255g に維持した。

4.オペラント行動試験用装置

実験にはラット用のレバーつきオペラント実験箱(室町機械、東京)を使用した。実験箱内前面のパネルに、反応用レバーが左右に二つ、その上に刺激提示用ランプ、ボックス中央上部にハウスライト、下部に給餌ボックスが組み込まれていた。ラットが課題に応じたレバー押し反応を行うことで報酬(実験用エサ 1 粒 45mg)が給餌ボックスの中に提示される。各実験箱を換気扇つきの防音箱の中に入れて実験を行った。レバー押し反応の記録、報酬の提示などは、コントロールボックス(室町機械と共同開発)にて管理し、10 ミリ秒単位で記録を行い、パーソナルコンピュータで制御した。

5. オペラント行動試験課題

各腹の雌雄 1-2 匹の仔動物を用い、オペラント行動試験を行った(コントロール群; 雄 9 匹・雌 9 匹、TCDD 曝露群; T50: 雌雄各 8 匹、T200: 雄雌各 8 匹、T800: 雄雌各 8 匹、PCB126曝露群; P500: 雄 8 匹・雌 6 匹、P2000: 雄雌各 8 匹、P8000: 雄雌各 8 匹)。12 週齢以降、これらのラットに対し、二種類のレバー押し課題を訓練した。一つは定率強化(Fixed Ratio, FR)課題で、一定の回数のレバー押し反応に対してエサ粒を一つ与える課題である。本研究では、FR20(20 回目のレバー押し反応にエサを提示、以下 FR20)課題を訓練した。もう一つの課題は、低率反応分化強化(differential reinforcement of low rates, DRL)で、直前のレバー押し反応から一定の時間経過後のレバー押し反応に対してエサを提示した。本研究では、DRL20 秒(レバー押しの反応間間隔が 20 秒以上あった場合にエサを提示、以下 DRL20)課題を訓練した。訓練期間は、FR20 および DRL20 課題それぞれ 8 日間であった。その後、上記の 2 種類を組み合わせた、FR20DRL20 多元強化(Multiple reinforcement schedule、Mult)スケジュール)を連続 30 日間(1 日 1 セッション、49 分間)行った。Mult スケジュールでは、1 セッション内で、FR20 と DRL20 課題をそれぞれ 2 分間と 5 分間交互に 7 回ずつ提示した(計 49 分間)。FR20 の時には刺激提示油ランプを点灯し、DRL20 の時には消灯した。

6.解析方法

母獣の体重増加率、産仔数、仔獣の体重増加率は繰り返しのある二元配置の分散分析にて解析を行った。同腹から生まれた仔の雌雄の比率については、m×n の比率検定を用いて解析を行った。Mult FR20DRL20 スケジュールにおける曝露影響は、それぞれの課題ごとに、1 分間あたりのエサの数(報酬獲得率)、1 分間あたりの反応数(反応率)を算出し、繰り返しのある二元配置の分散分析法にて解析した。

C. 研究結果

母獣の体重増加率、産仔数、仔獣の体重増加率、雌雄仔の比率については、TCDD および PCB126 曝露全群とコントロール群との間に差はみられなかった。低用量 TCDD および TEQ 相当量の PCB126 の母体曝露により、成熟後の仔動物においてオペラント行動試験の成績に影響があらわれた。

(1) TCDD 曝露群について

FR20 の反応率においては、コントロール群に比べ T200 群が有意に高率の反応を示した。報酬獲得率は、コントロール群に比べ T50 群が有意に少なく($p<0.01$)、T200 群が有意に多かった($p<0.01$)(図 4)。T800 群

では有意差は見られなかったものの、報酬獲得率が低下する傾向がみられた。DRL20での反応率は、T200 群がコントロール群よりも有意に高かった($p<0.01$)。DRL20 での報酬獲得率は、TCDD 曝露全群がコントロール群よりも有意に低かった(それぞれ$p<0.01$)(図 5)。

（2）PCB126 曝露群について

FR20 の反応率は、P8000 群で有意に低下した($p<0.01$)。報酬獲得率は、コントロール群に比べ P2000 群が有意に多く、P8000 群において有意に少なかった(それぞれ$p<0.01$)。DRL20 の反応率については、曝露全群ともコントロール群との間に差はみられなかった。報酬獲得率においては、P2000 群がコントロール群に比べて多くの報酬を獲得していた($p<0.01$)。

（3） TCDD と PCB126 曝露群との比較

TCDD 曝露群と、各 TCDD の曝露量の TEQ に相当する PCB126 曝露群をそれぞれ比較した。FR20 においては、T200 群と P2000 群との間に反応率と報酬獲得率に差が認められ(それぞれ $p<0.01$)、T200 群が有意に高率反応を示し、報酬もより多く獲得していた。また、T800 群と P8000 群との間にも反応率および報酬獲得率に差が認められた(それぞれ $p<0.01$)。T800 群が高率反応を示し、より多くの報酬を獲得していた。DRL20 においては、T200 ng/kg 群と P2000 群との間に反応率に差が認められ($p<0.01$)、T200 群が有意に高率反応を示した。報酬獲得率では、T50 群と P500 群との間に差が認められ($p<0.01$)、T50 群は、P500 群と比べると報酬獲得率が有意に低かった。

D. 考察

母体に対する低用量の単回曝露が、仔動物における成熟後のオペラント行動に影響を及ぼし、その結果記憶・学習機能が部分的に障害されたことが強く示唆された。オペラント行動に対する影響は、TCDD および PCB126 ともに、曝露用量依存的ではなく、曝露用量特異的な影響としてあらわれた。また、本実験の結果から、TCDD と PCB126 がオペラント行動に対して異なる影響を及ぼした可能性も示唆された。

（1）TCDD の影響について

TCDD 曝露後、妊娠経過、母獣の体重増加率、仔獣の生育経過、体重増加に影響がなかったにもかかわらず、オペラント行動試験においては最低用量(50ng/kg)曝露群においても有意な影響が確認された。母体に対する低用量の TCDD 曝露により、仔動物における成熟後のオペラント行動に影響が見られたことは、記憶･学習機能がわずかな量の TCDD 曝露に対しても非常に敏感に反応したことを表している。T200 群では FR20 で高率反応をした結果報酬獲得数が亢進した。FR20 における反応率が逆 U 字型を示したことは、TCDD の毒性がオペラント行動に対して用量特異的な作用を及ぼす可能性を示している。一方、T200 群では DRL20 でも同様に高率反応し、報酬獲得率は低くなった。この結果は、T200 の FR20 における学習能力が亢進されたわけではなく、反応が亢進したこと、そして二つの課題に対する弁別学習が成立しなかったことを示している。また、T50 および T800 群における DRL20 の反応率は、ほぼコントロール群と同率であったにもかかわらず、報酬獲得率は有意に低かった。T50 および T800 は、DRL20 でコントロール群とは異なる反応をした結果、少ない報酬しか獲得できなかったと考えられる。言い換えると、反応と報酬との関係、つまり DRL20 における最適反応を学習できなかった可能性がある。

（2）PCB126 の影響について

FR20 では、P2000 および P8000 群において、反応率および報酬獲得率ともにコントロール群と比較して有意差が見られた。しかしながら、反応は用量依存的ではなく、

P2000 群は高率反応を示したが、P8000 群は低率反応を示した。DRL20 においては、反応率に有意差は見られなかったが、報酬獲得率では P2000 群がコントロール群よりも低率を示し、最適反応の学習が獲得できなかったことが示唆された。

（3）TCDD と PCB126 の毒性比較

TCDD と PCB126 曝露による影響は、FR20 において両者とも曝露用量依存的ではなく、本実験で用いた中間曝露用量(T200、P2000)群で反応が亢進し、最低用量((T50、P500)群および最高用量(T800、P8000)群で反応が減少する逆 U 字型となった。TCDD および PCB126 がレバー押し反応におよぼした毒性は、毒性等価係数と同等、もしくは若干低めの値が適当なことを示唆する結果と考えられる。

しかしながら、TCDD では最低曝露用量で影響が出ていた(FR20 の反応率と報酬獲得率)のに対し、低下傾向はみられたものの、PCB126 では有意差が見られなかった。また、TCDD では最高用量では影響が認められなかったが、PCB126 ではコントロール群と比べ、反応率・報酬獲得率が有意に低下した。

E. 結論

TCDD あるいは PCB126 それぞれの発達期曝露が、成熟後の記憶・学習機能に影響を及ぼした。TCDD のオペラント行動に対する毒性は曝露用量依存的ではなく、逆 U 字パターンの用量特異的な影響としてあらわれた。PCB126 の毒性にも用量依存関係は見られず、TEF と同様か若干低めの値を示唆する「TCDD 様毒性」を示した。さらに最高用量曝露群では、PCB126 は TCDD よりも強い毒性も示したことから、PCB126 の「非 TCDD 毒性」の存在も示唆された。

F. 参考文献

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G. 健康危険情報　特に無し

H. 知的財産権の出願・登録状況

1. 特許取得
   特に無し
2. 実用新案取得
   特に無し
3. その他
   特に無し

Ⅰ． 図の説明

図 1.　FR20 課題における反応率
　FR20DRL20Mult スケジュールにおける
FR20 課題の 1 分間あたりの反応数
*<0.05(vs. control)、 † <0.05。

図 2.　FR20 課題における報酬獲得率
　FR20DRL20Mult スケジュールにおける
FR20 課題の 1 分間あたりの報酬獲得数。
*<0.05(vs. control)、 † <0.05。

図 3.　DRL20 課題における反応率
　FR20DRL20Mult スケジュールにおける
DRL20 課題の 1 分間あたりの反応数。
*<0.05(vs. control)、 † <0.05。

図 4.　DRL20 課題における反応率
　FR20DRL20Mult スケジュールにおける
DRL20 課題の 1 分間あたりの報酬獲得数。
*<0.05(vs. control)、 † <0.05

図１

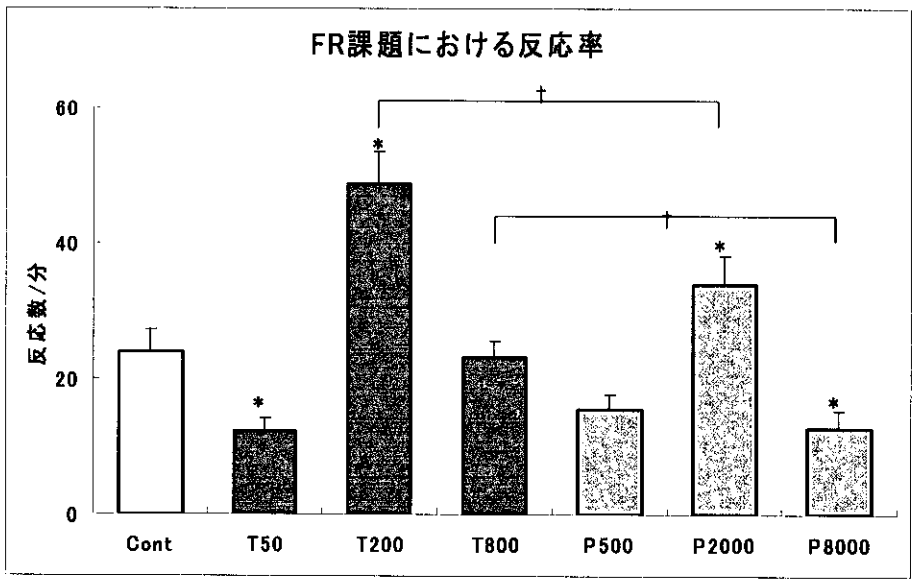

FR課題における反応率
反応数/分
60
40
20
0
Cont
T50
T200
T800
P500
P2000
P8000

図2

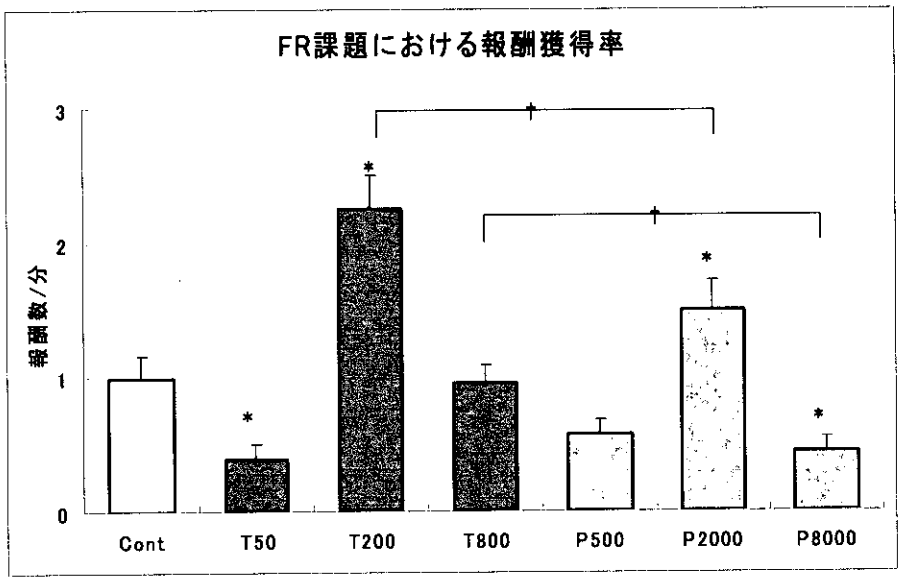

FR課題における報酬獲得率
報酬数/分
3
2
1
0
Cont
T50
T200
T800
P500
P2000
P8000

図3

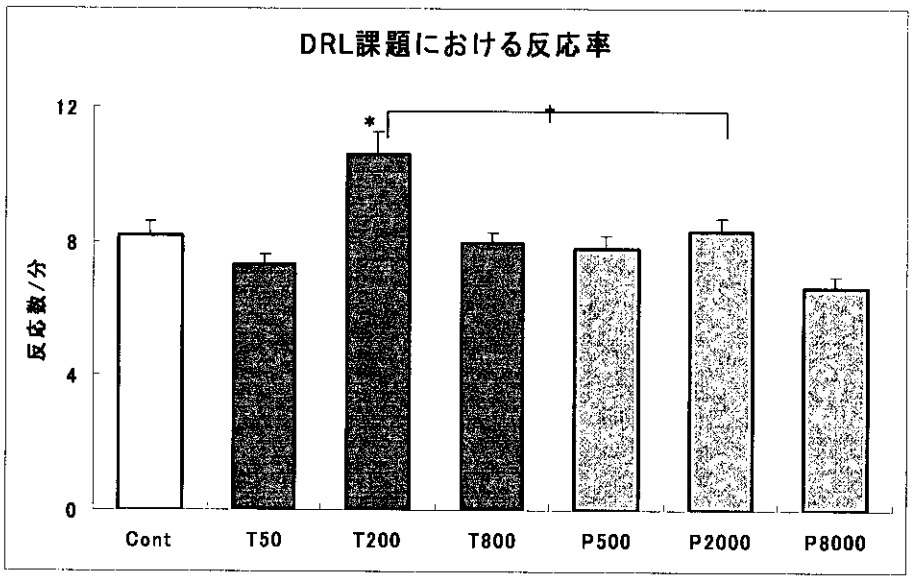
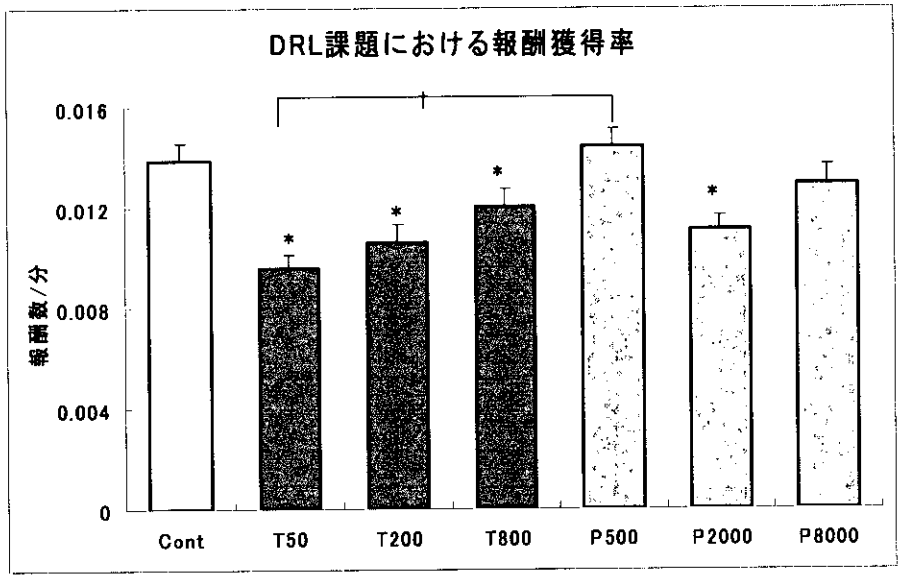

DRL課題における反応率
反応数/分
12
8
4
0
*
†
Cont
T50
T200
T800
P500
P2000
P8000

図４


DRL課題における報酬獲得率
0.016
0.012
0.008
0.004
0
報酬数/分
Cont
T50
T200
T800
P500
P2000
P8000

Ⅲ. 研究成果の刊行に関する一覧表

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Wu Q., Ohsako S., Ishimura R., Suzuki J. S., and **Tohyama C.**	Exposure of Mouse Preimplantation Embryos to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Alters the Methylation Status of Imprinted Genes *H19* and *Igf2*	Biol. Reproduction	in press	2004
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Ishizuka M., Yonemoto J., Zaha H., **Tohyama C.**, and Sone H.	Perinatal exposure to low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters sex-dependent expression of hepatic CYP2C11	J. Biochem. Toxicol.	17: 278-282	2003
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Nishimura N., Yonemoto J., Miyabara Y., Sato M., and **Tohyama C.**	Rat thyroid hyperplasia induced by gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-	*Endocrinology*	144: 2075-2083	2003

	dioxin			
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20031294

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J BIOCHEM MOLECULAR TOXICOLOGY
Volume 17, Number 5, 2003

Perinatal Exposure to Low Doses of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Alters Sex-Dependent Expression of Hepatic CYP2C11

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Received 12 February 2003; revised 10 June 2003; accepted 11 June 2003

ABSTRACT: The cytochrome P450 (CYP) isoform CYP2C11 is specifically expressed in the liver of adult male rats, and 5α-reductase is specifically expressed in the liver of the adult female rats. The sexually dimorphic expressions of these hepatic enzymes are regulated by the sex-dependent profiles of the circulating growth hormone (GH). However, it is not well known whether hormonal imprinting or activation factors in the neonatal brain influence the sexually dimorphic expression patterns of hepatic enzymes. We therefore examined the effect of perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on sex-dependent expressions of hepatic enzymes. Pregnant rats were treated with TCDD at a dose of 0, 200, or 800 ng/kg on gestation day 15, exposing the pups to the chemical. Although the expression of CYP2C11 protein in the livers of male pups on postnatal day (PND) 49 was significantly higher than that of the controls, but the 5α-reductase activities in the livers of female pups were not altered by exposure to TCDD. Focusing on perinatal periods, testosterone and estrogen levels significantly increased in the brain of male pups on PND 2. The results suggest that the alteration of testosterone and estrogen levels affect hormonal imprinting in the neonatal brain of male pups, and thus induces a change in the level of male-specific hepatic CYP2C11. We conclude that perinatal exposure to TCDD at low doses may change the sexual differentiation of the neonatal brain in male rats. © 2003 Wiley Periodicals, Inc. J Biochem Mol Toxicol 17:278–285, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10090

KEYWORDS: **TCDD; CYP2C11; Imprinting; Testosterone; Estrogen**

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs) are representative planar halogenated aromatic hydrocarbons (HAHs) or dioxins and related compounds. TCDD is the most toxic compound among the HAHs; it produces various toxic effects, such as body weight loss, thymic atrophy, dermal disorders, hepatic damage, carcinogenicity, teratogenicity, reproductive toxicity, immunotoxicity, and endocrine toxicity [1,2]. The toxicity of HAHs, including TCDD, is mediated through the aryl hydrocarbon (Ah) receptor. Numerous studies have reported that the Ah receptor interacts with the estrogen receptor and inhibits its effects [3–5]. The level of expression of CYP1A is regulated by the Ah receptor and induced by TCDD, which has high affinity to the Ah receptor [6].

Steroid hormones are synthesized and metabolized by the CYP monooxygenase system. CYP2C11, the predominant male-specific CYP isoform, is expressed in the liver of adult rats [7] and regulated by the sexually determined circulation profile of growth hormone (GH) [8]. CYP2C11 is not expressed in the liver of immature rats. The developmental induction of this male-specific CYP is imprinted by exposure to testosterone-derived estrogen during the neonatal period. The conversion of testosterone from estrogen is mediated by aromatase, CYP19 [7]. Testosterone metabolism is catalyzed mainly

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by CYP2C11 in the liver of male rats [9]. Therefore, the pattern of steroid hormone metabolism in the liver of male rats is thought to be important for the development of the reproductive organs and the brain.

Hepatic 5α-reductase activity increases after sexual maturation in female rats. 5α-reductase converts testosterone to dihydrotestosterone (DHT), which has high affinity for the androgen receptor [10]. DHT, however, does not affect the mature female liver, owing to the low expression of androgen receptors. 5α-Reductase is also regulated by the circulation profiles of GH after sexual maturation of the female brain [11].

Sex differences in the GH secretion pattern follow pituitary maturation at puberty and are imprinted by exposure to steroid hormones in the neonatal hypothalamus. The male GH pattern is characterized by a low basal hormone level with marked peaks every 3–4 h [12,13]. Female rats exhibit a higher basal hormone level without regular marked peaks. Therefore, hepatic steroid metabolism in the rat is sexually differentiated by the sex-dependent GH pattern, which is imprinted by the levels of steroid hormones in the neonatal brain.

We examined the expression of liver enzymes involved in the metabolism of steroid hormones during masculinization and feminization in rats, and hormone levels in neonatal tissues to elucidate whether prenatal exposure to TCDD affects hormonal imprinting in the brain of neonatal rats.

MATERIALS AND METHODS

Animals and Administration of TCDD to Pregnant Rats

Holtzman rats were purchased from Harlan Sprague-Dawley (USA). Rats were maintained in individual cages and kept under a photoperiodic cycle of 12 h light/12 h dark in an air-conditioned animal room, at 22 ± 3°C and 55 ± 10% relative humidity. Rats received commercial MF chow (Oriental Bio Service Kanto Inc., Tokyo, Japan) and tap water ad libitum. Male and female rats (9 weeks old) were mated at a ratio of one male to one female overnight under standard laboratory conditions. After confirmation of pregnancy by observation of the vaginal plug, the females were separated and housed in individual cages. All animals were treated with chemicals and sacrificed between 10:00 AM and 12:00 noon.

TCDD (Cambridge Isotope Laboratories, Andover, MA) was dissolved in *n*-nonane (Sigma, St.Louis, MO) at a concentration of 20 μg/mL. The TCDD/*n*-nonane solution was diluted in corn oil so that the desired dose of 2.5 mL/kg could be delivered. The pregnant rats were administered 0, 200, or 800 ng TCDD/kg body weight by gavage on gestation day (GD) 15. Pups were killed on postnatal days (PNDs) 2, 5, 21, or 49. The liver, reproductive organs, and brain were removed and stored at −80°C until use.

Preparations of S9 and Microsomal Proteins

The livers were homogenized in 3 volumes of 0.1 M potassium buffer (pH 7.4). Each homogenate was centrifuged at 9,000 × *g* for 20 min [14], and the supernatant was then centrifuged at 105,000 × *g* for 1 h. Each pellet was homogenized with 25 mL of the potassium buffer, and then centrifuged at 105,000 × *g* for 1 h. The microsomes thus obtained were homogenized with potassium buffer, frozen in liquid nitrogen, and then stored at −80°C until the assays for immunoblotting and enzyme activities. The protein concentration was determined by the method of Lowry et al. [15] using bovine serum albumin as a standard.

Immunoblot Analysis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli [16] using 10% polyacrylamide separation gel. After electrophoretic transfer onto nitrocellulose filters, the CYP forms were characterized with CYP2C11-specific antibody (Daiichi Pure Chemicals, Tokyo, Japan) and detected with an ECL system (Amersham Pharmacia Biotech, Tokyo, Japan). Spectral configurations of the immunoblots were analyzed using the NIH Image computer program [17].

Determination of Testosterone 5α-Reductase Activity

The activity of testosterone 5α-reductase was determined by essentially the same method as described by Lax et al. [18]. Enzymatic activities were determined under the linear range, including the reaction time, substrate, and protein concentrations. The reaction mixture for the assay consisted of 100 mM potassium phosphate buffer (pH 7.4), 0.5 mg S9 protein, an NADPH-generating system (final concentrations: 0.5 mM NADPH, 10 mM glucose-6-phosphate, and 4 mM magnesium chloride), and substrate (250 μM testosterone) in a final volume of 1.0 mL. The reaction was started by incubating 5α-reductase with testosterone in the liver microsome fraction for 5 min.

Determination of CYP2C11 mRNA by Semi-Quantitative RT-PCR

The expression levels of CYP2C11 mRNA in the rat liver on postnatal day 21 were determined by the semi-quantitative RT-PCR method. Total RNA was isolated from rat liver using Isogen (Nippon Gene,

Toyama, Japan). For conversion of total RNA to cDNA, a 20-μl reaction mixture containing reverse transcriptase was prepared according to the manufacturer's instructions (TaKaRa, Osaka, Japan). For the PCR amplification of cDNA, primers for CYP2C11 and cyclophillin, as an internal standard, were purchased from TaKaRa. PCR reactions were carried out in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, San Diego, CA) using denaturing, annealing, and extension cycling conditions of 94°C for 10 s, 56°C for 20 s, and 72°C for 1 min. All amplifications were carried out for 35 cycles. Amplified cDNA products were separated on 1% agarose gel. Gels were stained with ethidium bromide and photographed on an UV transilluminator. The staining intensity was determined by NIH Image software.

Determination of Steroid Hormones

Commercial ELISA kits were used to determine the levels of steroid hormones in reproductive organs, serum, and brain. Estrogen in serum was measured with a 17β-estradiol ELISA kit (Takeda Health Care Company, Tokyo, Japan). Estrogen in ovaries and brains was determined with an estrogen ELISA kit (Takeda), which detects estron, estriol, and estradiol. In the determination of androgen, a specific detection kit for testosterone (Cayman Chemical Company, Ann Arbor, MI, USA) was used. Hormones were extracted by adding 10 vol of diethyl ether to the serum or tissues, followed by homogenization. Aliquots were thoroughly mixed with a vortex mixer. The ether phase was removed by evaporation at 37°C with a gentle stream of dry nitrogen. The extract was redissolved in 10% methanol, and the sample was added to a 96-well plate. The levels of steroid hormones were determined according to the manufacturers' instructions.

Statistical Analysis

The results are presented as mean values ± SD. Differences in means were assessed by analysis of variance (ANOVA), followed by Scheffé's post-hoc and Dunnett's tests for immunoblotting analysis, mRNA expression levels and determination of enzymatic activity, and by Student's t test for steroid hormones. P values less than 0.05 were considered statistically significant.

RESULTS

Alterations in CYP2C11 and Testosterone 5α-Reductase

Immunoblotting analysis using anti-CYP2C11 antibody showed that the perinatal exposure to TCDD at a dose of 800 ng/kg produced a remarkable increase in CYP2C11 in the liver of male rats (Figure 1). No change was observed in CYP2C11 expression on PND 21. However, the expression level of CYP2C11 on PND 49 was three times higher than in the controls. We also determined hepatic CYP2C11 mRNA expression by semi-quantitative RT-PCR on PND 21 because the protein expression of sex-specific hepatic enzymes in general is low in prepubertal male rats. As shown in Figure 2, the CYP2C11 mRNA on PND 21 was expressed at higher levels in TCDD-treated livers than in those of control animals, and was also found to increased dose-dependently by perinatal exposure to TCDD. CYP1A1 mRNA expression levels in TCDD-treated rat liver on PND 49 were also higher than those of controls in the present study (data not shown).

We next determined the level of testosterone 5α-reductase activity in the liver of the female offspring born to TCDD-exposed rats. The activity in the livers of TCDD-exposed rat offspring tended to be suppressed in comparison to that of the control females, although without a statistical difference (Figure 3).

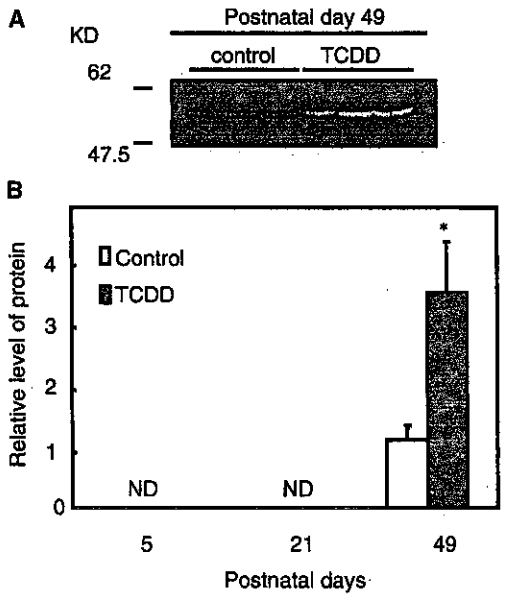

FIGURE 1. Expression of CYP2C11 in the liver of male rats. Effects of TCDD on the expression levels of CYP2C11 were determined by Western blotting. Liver microsomes from male rats on PNDs 5, 21, and 49 were collected and placed on acrylamide gel. Anti-rat CYP2C11 antibody was used to detect the expression levels of CYP2C11 apoprotein. (A) Electrophoresis of the Western blot on PND 49. (B) The graph represents band intensities of the Western blot. Values represent the mean ± SD of three samples. Open bars: control; closed bars: 800 ng TCDD/kg. *: Significantly different at $P < 0.05$ from corresponding control.

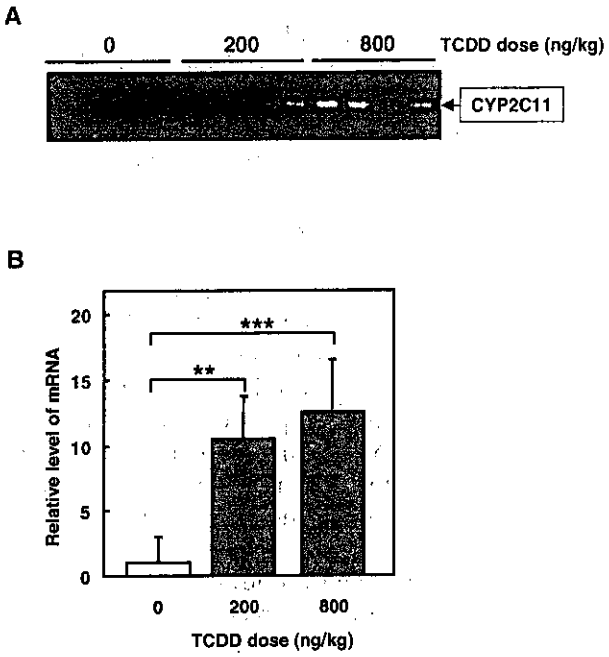

FIGURE 2. Effects of TCDD on expression of CYP2C11 mRNA in liver on PND 21. The expression levels of CYP2C11 mRNA in the liver of male rats exposed to TCDD at 0, 200, or 800 ng/kg on PND 21 were determined by semi-quantitative RT-PCR. (A) Electrophoresis of RT-PCR products. (B) Intensities of ethidium bromide staining of electrophoresis. Values represent mean ± SD of four samples. ***, **: Significantly different at $P < 0.001$, $P < 0.01$, respectively, from control.

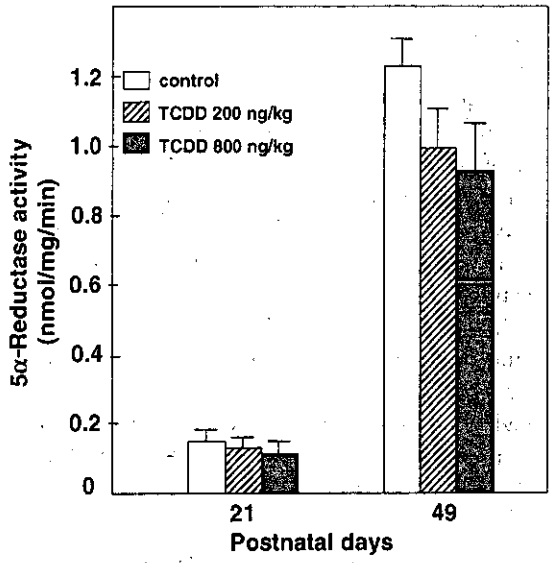

FIGURE 3. Testosterone 5α-reductase activity in liver microsomes of TCDD-exposed female rats. Microsomes were obtained from the liver on PNDs 5, 21, and 49. Values represent the mean ± SD of three samples. Open bars: control; closed bars: 200 and 800 ng TCDD/kg.

Sex-Steroid Hormone Levels in Serum, Reproductive Organs, and Brain

The levels of hormones in serum, reproductive organs, and brain of rats on PND 5 are shown in Figures 4, 5, and 6, respectively. The serum estrogen and testosterone levels were not significantly affected by TCDD exposure (Figure 4), although the 17β-estradiol concentration is suppressed in adult female rats by a high TCDD dose [19,20].

In the reproductive organs and brain, we determined total estrogens including estron, estriol, and estradiol by ELISA because the metabolism of estradiol is relatively fast in the ovary and the amount of estradiol in the brain is very low. There was no difference in ovarian estrogens between female pups exposed to TCDD and those not exposed, but testosterone concentrations in the testes of TCDD-treated males were affected by TCDD treatment (Figure 5).

Circulating steroid hormones reach the brain. Testosterone is converted to estrogens in the neonatal brain [21]. Figure 6 presents the levels of hormones in the brain on PND 2. In the females, perinatal exposure to TCDD did not significantly affect levels of either estrogens or testosterone. In contrast, it significantly increased the testosterone and estrogen level in males.

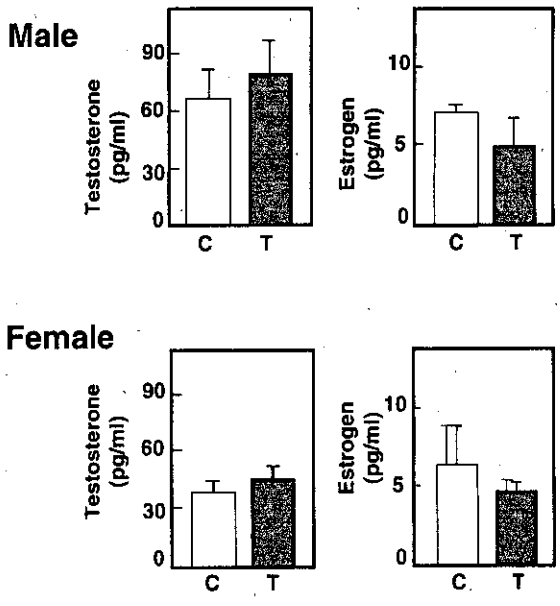

FIGURE 4. Sex-steroid hormones in serum. The levels of steroid hormones in the serum of TCDD-exposed rats were determined by ELISA. The serum was collected from neonatal pups on PND 5. Upper panel: male; lower panel: female. C: control, T: 800 ng TCDD/kg. Values represent the mean ± SD of 11–13 samples.

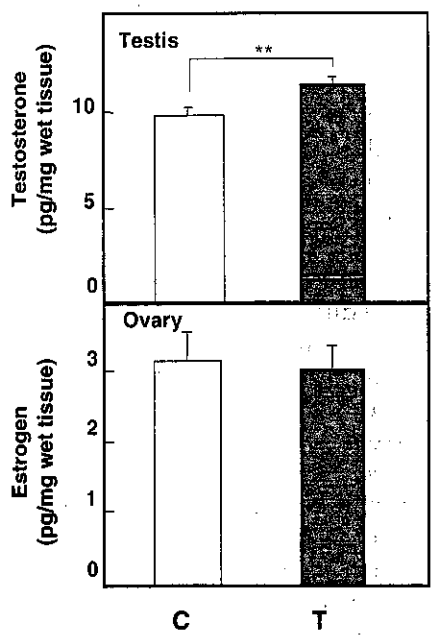

FIGURE 5. Sex-steroid hormones in reproductive organs. The effects of TCDD on the synthesis of steroid hormones in the testis and ovary of rats on PND 5 were determined by ELISA. Upper panel: testosterone; lower panel: estrogens. C: control, T: 800 ng TCDD/kg. Values represent the mean ± SD of 3–5 samples. **: Significantly different at $P < 0.01$ from control.

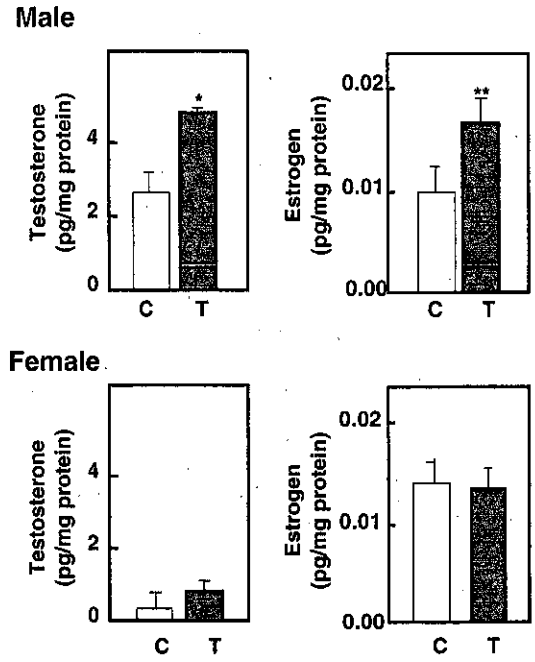

FIGURE 6. Sex-steroid hormones in brain. The effects of TCDD on the levels of steroid hormones in the brain of rats were determined by ELISA. Brains were collected from neonatal pups on PND 2. Upper panel: males; lower panel: females. C: control, T: 200 ng TCDD/kg. Values represent the mean ± SD of 4–6 samples. *, **: Significantly different at $P < 0.05$, $P < 0.01$, respectively, from control.

DISCUSSION

Our previous reports have shown that exposure to TCDD during fetal and lactational period influences sexual dimorphism in sweet preference or the male sexual behavior after sexual maturation [22,23]. CYP1A1 mRNA, a sensitive marker in the liver of rodents exposed to TCDD, was significantly induced by the same protocol of exposure on PND 49 [24]. Large amounts of TCDD were also detected in livers of offspring on PND 49 [24]. In the present study, CYP1A1 mRNA expression levels in TCDD-treated rat liver were higher than those of controls on PND 49, as the same as reported previously [24]. Thus, the effect of TCDD exposure during the fetal and lactational periods continues until sexual maturation. Therefore, we had a working hypothesis that residual TCDD caused the overexpression of CYP2C11 protein measured on PND 49.

Since the protein expression of sex-specific hepatic enzymes in general is low in prepubertal male rats, we determined hepatic CYP2C11 mRNA expression by semi-quantitative RT-PCR on PND 21. The CYP2C11 mRNA was expressed at higher levels in TCDD-treated livers than in those of control animals. The level of CYP2C11 mRNA expression was dose-dependently increased by perinatal exposure to TCDD. However, the expression levels of CYP2C11 decreased in the liver of adult rats treated with the Ah receptor agonist 3-methylcholanthrene, which is a polyaromatic hydrocarbon (PAH), after sexual maturation [25,26]. Exposure to another Ah receptor agonist, benzo[*a*]pyrene, which is also a PAH or TCDD, suppressed the expression level of CYP2C11 in cultured hepatocytes [27,28]. Thus, in our study we relinquished the above hypothesis and speculated that the residual TCDD does not directly induce the overexpression of CYP2C11 in liver on PND 49.

Fujita et al. [29] also reported elevation of the CYP2C11 expression level in the liver of mature rats treated with benzo[*a*]pyrene, a PAH, during the neonatal period. However, neonatal exposure to tamoxifen decreased expression of hepatic CYP2C11 protein in addition to reducing testicular weight [30]. Our previous report showed that perinatal exposure to TCDD did not alter testicular weight of male rats at 65–120 days of age [31]. We remark that perinatal exposure to TCDD or other Ah receptor (AhR) agonists during the neonatal imprinting results in the up-regulation of hepatic CYP2C11 expression after sexual maturation. It is suggested that AhR-mediated signals affect the hormonal imbalance during the neonatal imprinting periods.

Sexual differences in the brain arise through neonatal exposure to steroid hormones, testosterone and estrogen, from the gonads. Therefore, we determined the

levels of steroid hormones in the serum, gonads, and brains of neonatal pups. In the females, levels of estrogen and testosterone were not significantly changed in any tissue. This lack of effect on sex steroid hormones in females reflects the fact that 5α-reductase activity was not affected by the perinatal exposure to TCDD in the livers of female rats. Effects of exposure to TCDD on estrogen can be seen only at higher doses in adult females [19,20].

In contrast, the levels of testosterone in the testes of neonatal pups were significantly increased and the serum testosterone tended to be increased, but not significantly, by TCDD exposure in the present study. During PND 21–70, it was reported that elevations of serum testosterone were not statistically significant in TCDD exposed pups [32–34]. Our previous study also examined that the serum levels of pups on PND 49 was not significantly influenced by TCDD exposure [31]. Contrary, Haavisto et al. [35] reported that testosterone levels in serum were significantly elevated and testicular testosterone content was slightly increased (but not significantly) in pups on PND 19.5. These controversial results in the weaning phase and after are still unclear, but it can be speculated that there might be the specific period when TCDD affect circular and testicular levels of testosterone. Furthermore, we demonstrated the effects of TCDD on expressions of 5α-reductase and androgen receptor in the ventral prostate, and on the alterations of the external genital organs including ventral prostates at PND 49 and after [31,36]. Thus, effects of TCDD on testosterone synthesis or metabolism may induce alteration of sexual maturation in male rats.

To confirm that exposure to TCDD increased the level of testosterone in the testes and then affected testosterone imprinting, we determined the levels of testosterone and estrogens in the neonatal brains of males. The levels of both steroids were significantly increased. Thus, this evidence suggests that the changes of testosterone metabolism in the neonatal brain influenced the hepatic CYP2C11 induction after masculinization. In the present study, we used the total brain homogenate to detect the steroid hormones. However, the regional production and effects of steroid hormone are important for neonatal imprinting. Further study is needed to clarify this point.

Testosterone is synthesized from androstenedione by 17β-hydroxysteroid dehydrogenase, and is metabolized to dihydrotestosterone by 5α-reductase or is converted to 17β-estradiol by CYP19 [21]. The 5α-reductase activity in brain is high during perinatal development, and decreases with age and distributes in the hypothalamus as well as in other brain regions composed of white matter fibers [38]. The 5α-reductase type 2 mRNA increases after GD 18, peaks on postnatal day 2, then decreases gradually, suggesting that this pattern of expression appears to be correlated with testosterone synthesis in the testis [39]. CYP19 plays a critical role in sexual differentiation in the brain of rodents. The regional distribution of CYP19 mRNA in the preoptic/hypothalamic area of the perinatal brain closely regulates testosterone and estradiol levels, which leads to sexual differentiation in the brain [38]. Therefore, we determined 5α-reductase type 2 and CYP19 mRNA expression levels in perinatal brain on GD 20. There are no significant differences in both gene expression levels between the control and TCDD-treated male animals at doses of 100, 200, and 400 ng/kg (data not shown). Only the high dose of 1600 ng TCDD/kg suppressed the 5α-reductase expression. However, our previous work demonstrated that CYP19 expression and its aromatase activity in the brain of rats on GD 20 or on postnatal day 2 were decreased by prenatal exposure to TCDD [22], suggesting that TCDD slightly influences testosterone metabolism in the developing brain of rats. In addition, exposure to coplanar PCBs and PCDDs altered the activity of CYP19 in endometrial carcinoma cells and the hypothalamus [40]. This report supports our present result. Thus, it suggests that the changes of testosterone metabolisms in the neonatal brain cause the overexpression of CYP2C11 in the liver of mature male rats.

The expression of CYP2C11 in sexually matured liver is mainly regulated by GH profiles, which are modulated by releasing testosterone from the testes and by conversion to estradiol in the brain during the neonatal period. Thus, one of the possibilities suggested is that the changes of testosterone metabolism in the neonatal brain cause the overexpression of CYP2C11 in the liver of mature male rats. Further studies are needed to clarify whether or not alteration of testosterone metabolism or other factors directly contribute to the disruption of the regulation of CYP2C11 expression in mature liver.

In the current study, we concluded that environmental pollutants such as dioxins or other PHAHs could influence hormonal imprinting during the fetal and neonatal periods.

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