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Perinatal Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Alters Activity-Dependent Expression of BDNF mRNA in the Neocortex and Male Rat Sexual Behavior in Adulthood

Masaki Kakeyama[1,3,*], Hideko Sone[2,3], Yuichi Miyabara[1,3,†], Chiharu Tohyama[1,2,3]

[1]*Environmental Health Sciences Division, National Institute for Environmental Studies, Onogawa 16-2, Tsukuba 305-8506, Japan*

[2]*Endocrine Disruptor and Dioxin Research Project, National Institute for Environmental Studies, Tsukuba, Japan*

[3]*CREST, Japan Science and Technology Corporation, Kawaguchi, Japan*

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Abstract

Dioxin and its related compounds are suspected to cause neurological and nueroendocrinological disruption in human and laboratory animal offspring upon in utero and lactational exposure during growth and development. We tested the hypothesis by utilizing Long-Evans Hooded rats that perinatal exposure to dioxins affects the neocortical function and expression of sexual behavior in adulthood. In the sexual behavior test, perinatal exposure to TCDD significantly reduced the number of mounts and intromissions. The mRNA semi-quantification in in situ hybridization showed that the mating stimulus in control males induced c-fos mRNA expression in the preoptic area (POA) and the brain derived neurotrophic factor (BDNF) mRNA upregulation in the frontal cortex. In contrast, perinatal exposure to TCDD lowered the upregulation of BDNF mRNA in the frontal cortex but not that of c-fos mRNA in the POA. The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) was not affected. The results suggest that perinatal TCDD affects the neocortical function independently from the brain sexual differentiation and alters the expression of sexual behavior.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Sexual behavior; BDNF; c-*fos*; Frontal cortex; Preoptic area

INTRODUCTION

Dioxins (chlorinated dibenzo-*p*-dioxin congeners and related compounds including coplanar PCBs) have been found to be the most toxic environmental chemicals created by manufacturing and incinerating processes, and they tend to persist in the environment for long periods. Exposure to levels of dioxin in excess of the current environmental level has been reported to increase the risk of cancer and induce reproductive dysfunction in humans and animals (for review, see Schecter, 1996; Mukerjee, 1998; Birnbaum and Tuomisto, 2000; Petroff et al., 2001).

Moreover, because dioxins can be transferred transplacentally and lactationally from a mother to developing-offspring, developmental adversity in the offspring, such as neurotoxicity, is a concern even when a mother has been exposed to a relatively low level of dioxins that would not affect herself. Mental retardation and reduced learning ability in children have been reported to be associated with certain levels of exposure of their mother to dioxins (Schantz et al.,

* Corresponding author. Tel.: +81-298-50-2767; fax: +81-298-50-2574.
E-mail address: kake@nies.go.jp (M. Kakeyama).
† Present address: Research and Education Center for Inland-water Environment, Shinshu University, Nagano 392-0027, Japan.

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1996) and in experimental animals, maternal exposure to dioxins has been found to affect performance of learning behavior in monkeys (Schantz and Bowman, 1989) and rats (Seo et al., 2000).

In addition, based on behavioral results showing that a single oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic congener of dioxins, to pregnant female rats on gestational day 15 inhibited sexual behavior in offspring male rats (Mably et al., 1992; Gray et al., 1995), it has been suggested that certain levels of exposure to dioxins have adverse effects on the brain masculinization. This mode of exposure is known to cause in utero and lactational exposure of the offspring to TCDD during the perinatal period (Hurst et al., 2000; Ohsako et al., 2001), which is a critical period for masculinization of the brain.

These behavioral reports suggest that the fetal brain is highly sensitive to dioxin, and that developmental exposure to dioxin can cause permanent or semi-permanent the brain dysfunction. In addition, it is suggested that there are two types of effects resulting from in utero and lactational exposure to a low dose of dioxins: an effect on the brain sexual differentiation, principally in the preoptic area (POA)-hypothalamic axis, and an effect on the advanced brain function, principally in the neocortical axis. While those reports indicate the effect of low doses of dioxin on the brain of the offspring, as yet there is no evidence of effects at the morphological or molecular levels. In the previous report of the inhibition of brain masculinization, TCDD had no effect on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in male rats whose expression of sexual behavior was affected by TCDD (Bjerke et al., 1994).

We postulated that the assessment of the brain mechanisms regulating sexual behavior could serve a good model for determining the effects of environmental chemicals such as dioxins on the brain of offspring. There have been many reports about the brain mechanisms that regulate sexual behavior, and they have been mapped to many brain regions including the POA, the hypothalamus, the amygdala and the neocortex. The POA, hypothalamus and amygdala receive the hormonal and olfactory information (Arai et al., 1978; Kondo, 1992), and the neocortex is assumed to receive visual and auditory information and facilitate the expression of sexual behavior (Yamanouchi and Arai, 1992; Vega-Matuszczyk et al., 1993; Sachs, 1996; Swanson, 1992). We therefore thought that it would be possible to identify the effects of TCDD on the POA-hypothalamic axis and the neocortical axis by examining the effect on the brain mechanisms that regulate sexual behavior.

Here, we tested this hypothesis by the following experiments. First, we determined the effects of perinatal exposure to TCDD on expression of sexual behavior in male rats. Second, to clarify the effects of perinatal TCDD on sexual differentiation in the brain, we measured the volume of SDN-POA in male rats whose sexual behavior had been affected. Next we tested activity-dependent mRNA expression by semi-quantitative analysis of in situ hybridization histochemistry to examine the response of the brain (Morgan and Curran, 1989) to the mating stimulus in the POA and the frontal cortex, which are representatives of the POA-hypothalamic axis and the neocortical axis, respectively. Expression of c-*fos* mRNA expression was used for as the indicator for activity-dependent response in the POA, because mating stimuli have been reported to induce *Fos* immunoreactivity in this area (Robertson et al., 1991; Levine et al., 1995; Coolen et al., 1996; Lumley and Hull, 1999). Expression of BDNF mRNA was examined in the frontal cortex because upregulation of BDNF mRNA by the mating stimulus was observed in this experiment.

MATERIALS AND METHODS

Animals and Treatments

Long-Evans Hooded rats were purchased from Charles River (Chicago, IL), and animals were handled with humane care according to the guidelines on laboratory animal experiments at NIES. The following administration and necropsies were all performed in the hazardous chemical regulation area at the NIES. Perinatal exposure to TCDD was as described in a previous study (Ohsako et al., 2001). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was purchased from Cambridge Isotope Laboratory (Andover, MA). On day 15 of gestation, pregnant females were dosed by gavage (2.5 ml/kg) with 0 (vehicle), 200, or 800 ng TCDD/kg (7–9 animals, respectively). One day after birth, five males and five females were culled from the litters to allow for similar lactational TCDD exposure. Offspring were weaned on postnatal day (PND) 21 and unisexually group-housed (four to five pups) (35, 40, and 45 males in vehicle-, 200 ng TCDD/kg-, and 800 ng TCDD/kg-exposed males, respectively).

One or two male offspring per litter (14 animals per treatment group) were castrated on PND 49. From PND 77 until the end of the study, testosterone (T) (Sigma) was administered by implantation of a silicone tube (two, 5-cm long each, 1.57 mm, i.d.; 3.18 mm,

o.d.) containing T, to maintain the circulation of testosterone concentrations in similar amounts among the individual males (CAST + T males). Two to three gonadally intact males per litter (21 animals per group) were also utilized (Non-CAST males).

Male sexual behavior was assessed on PNDs 87 and 97. Spontaneous activity in the same period as the sexual behavior test was determined on PNDs 100 and 110 (14 animals per group). Non-CAST animals were decapitated on PND 120, and brain samples were used to measure the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) and determine the TCDD concentration (14 and 6 animals per group, respectively). CAST + T males were used for the in situ hybridization study. Maternal/pup viability and body weight gain were monitored throughout the study. Testis weight was measured on PND 49 (time of castration in CAST + T males) and PND 120 (end of the test in Non-CAST males).

Sexual Behavior

The masculine sexual behavior testing was conducted from 21:00 (2 h into the dark cycle) to 4:00 h. In the behavior test, each male rat was individually placed in an observation cage (50 cm × 60 cm), and after 5 min of adaptation, the test was started by introducing an estrous female rat (ovariectomized and injected with 10 μg of estradiol benzoate and 0.5 mg progesterone 48 and 4 h, respectively, before the test). The receptive female rat was replaced by another every 10 min to diminish the influence of partner affinity, according to the methods described previously (Yamanouchi and Kakeyama, 1992).

The following parameters of male sexual behavior were recorded: number of mounts (mounts with pelvic thrusts) and intromission (mounts with intromissive pattern) (during the time from the introduction of the stimulant female to the first ejaculatory pattern); mount and intromission latency (time from introduction of the stimulus female to the first mount and intromission); ejaculation latency (time from the first intromission until ejaculation); and post-ejaculatory interval (time between the first ejaculation and the first intromission of the next ejaculatory series).

Spontaneous Activity

Spontaneous behavior was measured as motor activity with a SUPERMEX system (Muromachi Kikai, Tokyo, Japan) in an empty plastic cage (40 cm × 40 cm). Ambulation was scored by using a personal computer interfaced to rat body-temperature sensitive sensors. The activity score was obtained from 21:00 to 4:00 h, the same time period as the sexual behavior test.

Gas Chromatography/Mass Spectrometry (GC/MS)

The brain samples on PND 5 were prepared from another series of pregnant females (three females in each exposure group), and the TCDD concentration of each brain was determined ($n = 6$ in each group, two offspring per litter). The brain samples on PND 120 were obtained from Non-CAST males ($n = 6$ in each group), and three samples were collected to one for the analysis. TCDD concentrations were determined by using a JMS700 high-performance double-focusing mass spectrometer (JEOL, Tokyo, Japan) according to the methods described previously (Miyabara et al., 1999).

SDN-POA Volume

Brain samples (14 animals per group of Non-CAST males) were fixed with a 10% formalin solution, and serial frozen sections of 30 μm thick were stained with cresyl violet. The volume of the SDN-POA as defined by Gorski et al. (1980) determined by using a morphometric analysis system including a microscope (Q600 Imaging Workstation, Leica Microsystems, Rochester, NY).

In Situ Hybridization Study

On PND 120, CAST + T males (nine animals per group) were exposed to a mating stimulus in the same manner as the sexual behavior test. In this study, the stimulant females were rapidly removed after the first ejaculatory pattern. One hour after the start of the mating stimulus, the animals were sacrificed by decapitation, and their brains were quickly frozen with dry ice. Control males not exposed to a mating stimulus (five animals per group) were prepared for a comparison.

In situ hybridization histochemistry was performed as described in a previous study (Hashimoto et al., 1998). In short, brain sections were hybridized with DIG-labeled c-RNA probes (50 ng/ml), washed under high stringency conditions, and treated with RNase A (40 μg/ml). The hybridized strands were incubated with a polyclonal anti-digoxigenin antibody-alkaline phosphatase conjugate (1:10,000, Boehringer-Mannheim), and the resultant products were visualized

by alkaline phosphatase coloring reactions developed in a solution of nitroblue tetrazolium and X-phosphate.

For semi-quantification, the antisense cRNA probe and sense probe were each applied to two serial brain sections mounted side-by-side, and the grain density produced by the sense probe was regarded as the background. Four coronal sections (12 μm thick) were taken every 48 μm from the frontal cortex and the POA (from 3.6 to 3.8 mm and from 0.2 to 0.0 mm, respectively, anterior to the bregma) according to the brain atlas (Swanson, 1992). Grain density was quantified by the morphometric analysis system (Q600 Imaging Workstation, Leica Microsystems), and the grain signals were calculated in the unit area indicated by shadow in the drawings of the coronal brain section in Fig. 4A and E, the frontal cortex and the POA, respectively (6.0 mm^2).

Preparation of In Situ Hybridization Probe

The cRNA probes were synthesized by using a PCR II plasmid vector (Invitrogen, San Diego, CA) by the TA-cloning method from rat brain cDNA by PCR. The primers for the *c-fos* (489–1590) gene were 5-AGA GCG CAG AGC ATC GGC AG-3 (forward) and 5-AAA AGA GAC ACA GAC CCA GG-3 (reverse), and for the BDNF (73–836) gene were 5-CCT GTT CTG TGT CTG TCT CT-3 (forward) and 5-GTC TAT CCT TAT GAA CCG CC-3 (reverse).

These sequences were confirmed by using the fluorescence-based DNA sequencing system ABI PRISM377 (Perkin-Elmer, Foster, CA). Antisense and sense digoxigenin-labeled riboprobes were prepared by in vitro transcription using digoxigenin-11-UTP (Boehringer-Mannheim, Tokyo, Japan). The cRNA probes were cut down to a length of 100 nucleotides by alkaline hydrolysis.

Statistical Analysis

All statistical analyses were carried out by using the Stat View 4 statistical analysis program (Abacus Concepts, Berkeley, CA). A difference was considered significant when $P < 0.05$. Data for the incidence and numbers of behaviors were analyzed by the χ^2-test. Data for behavior latency were analyzed by the Mann–Whitney *U*-test. Other dose-response data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc test, while time-course data for sexual behavior and body weight were analyzed by two-way or repeated-measures two-way ANOVA.

RESULTS

TCDD Transfer to the Brain Tissue of Offspring and Alteration of Sexual Behavior

TCDD concentrations in the brain were measured on PND 5 and 120. GC/MS analysis clearly showed a dose-dependent increase in TCDD in brain tissues on PND 5 ($P < 0.05$, versus vehicle-exposed group, by one way ANOVA, Table 1), indicating that the TCDD administration had been successfully executed and that maternal TCDD was transferred to the brain tissue of the offspring. On PND 120, the TCDD concentrations had decreased to a level close to the detection limit (below 0.02 pg/g). There were no significant differences in brain weight (by one-way ANOVA).

In the sexual behavioral test, all male rats exhibited complete sexual behavior and ejaculatory patterns more than twice in the 30-min test, but alterations in the pattern of sexual behavior as a result of TCDD exposure were clearly observed in the number of mounts and intromissions. The number of mounts and intromissions was 50% lower in the 800 ng TCDD/kg group than in the vehicle group ($P < 0.01$, by χ^2-test, Table 2). Mount frequency (MF) and intromission frequency (IF) was also reduced by TCDD exposure ($P < 0.01$, versus vehicle-exposed group, by Scheffé's post-hoc test, Fig. 1). Mount, intromission, and ejaculation latency and post-ejaculatory interval were unaffected. These changes in sexual behavioral patterns caused by TCDD were observed in both the Non-CAST and CAST + T males (by the Mann–Whitney *U*-test). In the CAST + T males, the latencies of behavior in the second test were significantly shorter

Table 1
TCDD concentrations in the brain tissue from male offspring perinatally exposed to TCDD

Exposure (ng)	TCDD concentration (pg/g wet tissue)		Brain weight (mg)	
	PND 5[a]	PND 120[b]	PND 5	PND 120
0	<0.10	<0.02 <0.02	698 ± 42	2101 ± 90
200	10.5 ± 1.5[c]	0.09 0.05	681 ± 61	2040 ± 99
800	22.8 ± 1.8[c]	0.11 0.05	682 ± 32	2014 ± 101

[a] TCDD concentration on PND 5 was determined individually.
[b] Three brain samples on PND 120 were collected to one for the analysis. Brain weight on PNDs 5 and 120 were determined individually.
[c] Significant ($P < 0.05$) difference between vehicle- and TCDD-exposed groups.

Table 2
Effect of perinatal exposure to TCDD on male rat sexual behavior on PND 97[a]

	Non-CAST, TCDD (ng/kg)			CAST + T, TCDD (ng/kg)		
	0	200	800	0	200	800
Mount latency (sec)[b]	23 (11–26)	21 (11–31)	25 (5–28)	63 (25–91)	71 (45–100)	61 (33–87)
Intromission latency (sec)[b]	30 (18–66)	27 (19–54)	30 (8–60)	82 (50–112)	81 (51–215)	88 (44–101)
Ejaculation latency (sec)[b]	450 (388–542)	471 (365–540)	441 (377–551)	814 (745–922)	915 (756–1524)	822 (784–914)
Number of mounts[c]	16.6 ± 1.5	14.0 ± 2.1	8.4 ± 1.0[d]	18.2 ± 1.4	17.5 ± 1.4	10.2 ± 1.0[d]
Number of intromissions[c]	12.1 ± 1.8	11.0 ± 1.5	6.2 ± 1.0[d]	14.1 ± 1.9	14.0 ± 1.6	7.2 ± 0.9[d]
Post-ejaculatory interval (sec)[b]	295 (251–314)	305 (266–322)	301 (254–330)	298 (266–314)	301 (244–320)	311 (277–330)

[a] Sexual behavior was assessed on PNDs 87 (first test) and 97 (second test), and the data obtained on PND 97 are shown ($n = 21$ in Non-CAST males and $n = 14$ in CAST + T males).
[b] Mount latency, intromission latency, ejaculation latency, post-ejaculatory interval are represented as median (min–max). Statistical difference among each-exposed group was not found by the Mann–Whitney U-test ($P > 0.05$).
[c] Number of mounts, number of intromissions are represented as mean ± S.E.
[d] Significant ($P < 0.01$) difference between vehicle- and 800 ng TCDD/kg-exposed groups.

than in the first test ($P < 0.05$, by the Mann–Whitney U-test). These behavioral latencies were similar to the values in previous studies using Long-Evans Hooded rats (e.g. Thor and Flannelly, 1979; Lumley and Hull, 1999). The low doses of TCDD in this experiment did not seem to critically impair expression of male sexual behavior except for the reduced number of mounts and intromissions.

Effects of TCDD on Spontaneous Activity, Body Weight Gain, Testis Weight and SDN-POA Volume

Spontaneous activity in the same period as the sexual behavior test (21:00–4:00 h) was determined on the 3 and 10 days after the sexual behavior test. No significant differences in spontaneous activity in the same period as the sexual behavior test (21:00–4:00 h) were noted among the groups (Fig. 2A). The data obtained on PND 110 (data not shown) were not significantly different among groups and were similar to the data on PND 100 by one-way ANOVA and repeated-measures two-way ANOVA. These findings indicate that the changes in sexual behavior in the present study were not attributable to a deficit of motor activity.

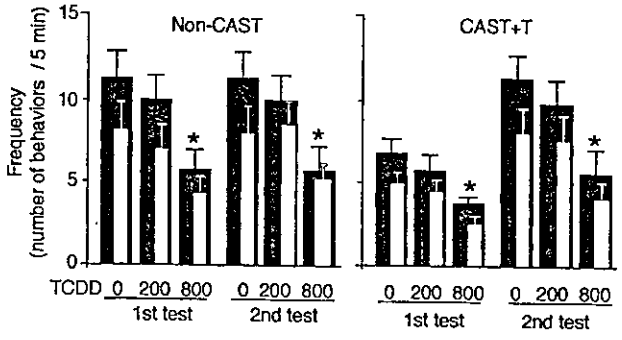

Fig. 1. Mount frequency (MF, solid bars) and intromission frequency (IF, open bars) in sexual behavior in male rat offspring perinatally exposed to TCDD. Frequency in each animal was calculated every 5 min from the start to the first ejaculatory pattern. Asterisks indicate that perinatal exposure to 800 ng TCDD/kg significantly reduced MF and IF (**$P < 0.01$, vs. vehicle-exposed group, by Scheffé's post-hoc test). Each bar represents a mean ± S.E. ($n = 14$ in CAST + T males, and $n = 21$ in Non-CAST males).

TCDD exposure in this experiment did not affect maternal body weight gain during pregnancy (by two-way and repeated measures of two-way ANOVA), maternal length (21 days of all animals), and maternal or pup viability (none of animals died throughout the test). All litters bred 14–17 offsprings, with no significant difference among groups (by two-way ANOVA). There was no significant difference in body weight gain of offspring from PND 1 to 42 (by two-way and repeated measures of two-way ANOVA). Exposure to TCDD at a dose of 800 ng/kg, however, caused a significant 8–10% reduction of body weight in the pups from PND 49 to 120 at the end of the study ($P < 0.05$, by Scheffé's post-hoc test, Fig. 2B). While testis weight in the 800 ng TCDD/kg group was also lower than in the vehicle group ($P < 0.05$, by Scheffé's post-hoc test), relative testis weight was unaffected by two-way ANOVA (Fig. 2C).

We next determined the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in Non-CAST males. The results were consistent with previous findings showing that the volume of the SDN-POA is 4.5 times larger in males than in females (Gorski et al., 1980). However, no significant difference in the volume of SDN-POA was found between the vehicle and TCDD groups by two-way ANOVA (Fig. 2D).

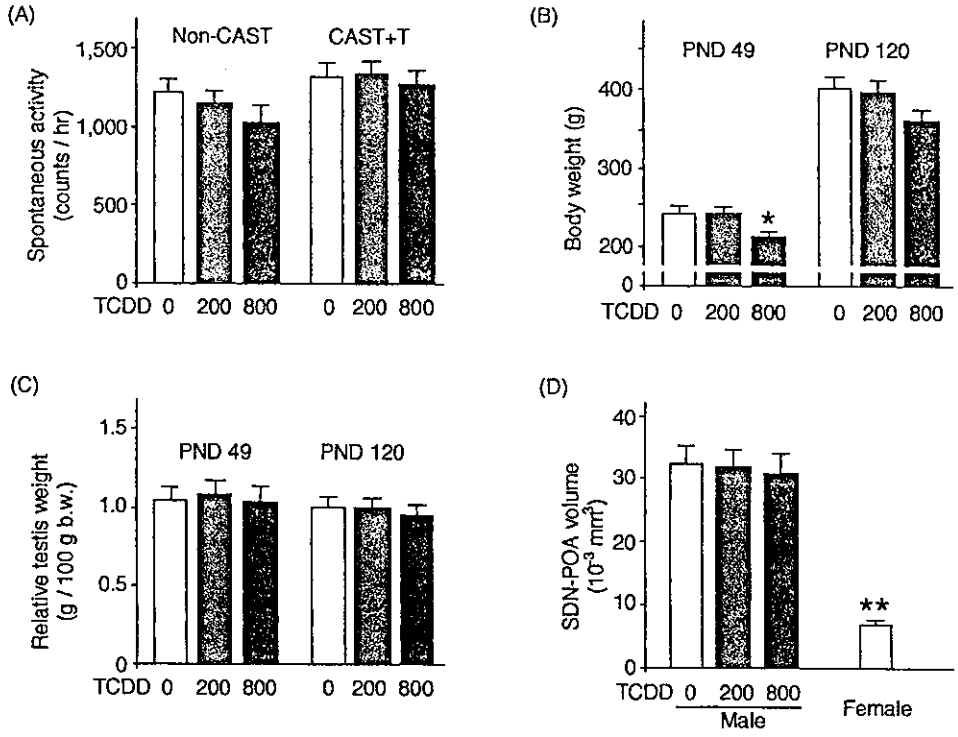

Fig. 2. Spontaneous activity, body weight, relative testis weight, and SDN-POA volume in male rat offspring perinatally exposed to TCDD. (A) TCDD did not affect the spontaneous activity score on PND 100 during the sexual behavior test (21:00–4:00 h). The data obtained on PND 110 (data not shown) were similar to those on PND 100 and no significance was found by one-way ANOVA and repeated-measures two-way ANOVA. Each bar represents a mean ± S.E. ($n = 14$ in CAST + T males, and $n = 21$ in Non-CAST males). (B) Body weight on PNDs 49 and 120 were reduced by 800 ng TCDD/kg exposure ($^*P < 0.05$, by Scheffé's post-hoc test). Each bar represents a mean ± S.E. ($n = 35$). There were no significant differences in body weight gain from PND 0 to 35 (data not shown). (C) Relative testis weight (testis weight/100 g body weight) on PNDs 49 and 120 was unaffected by perinatal exposure to TCDD. Each bar represents a mean ± S.E. ($n = 14$ for PND 49 and $n = 21$ for PND 120). (D) SDN-POA volume was unaffected by the perinatal exposure to TCDD, but the volume in males was 4.5 times larger than in females. Each bar represents a mean ± S.E. ($n = 14$).

Mating-Induced BDNF mRNA Upregulation and c-*fos* mRNA Induction

We performed semi-quantitative analysis and found that the density of the grains of in situ signal positively correlated with the relative amounts of the cRNA probes under non-saturated conditions (Fig. 3). This indicates that our in situ methodology faithfully reflects the amount of hybridization within the range of probe amounts.

Upregulation of BDNF mRNA by the mating stimulus in the vehicle-exposed animals was clearly detected in the frontal cortex. Although expression of BDNF mRNA was observed in the control males (no mating stimulus), the frontal cortex showed stronger in situ signals after the mating stimulus (Fig. 4A–C). In the semi-quantification, BDNF mRNA expression in the frontal cortex at 1 h after the mating stimulus was 400% higher than in the control males that were not exposed to the mating stimulus ($P < 0.05$, by Scheffé's post-hoc test, Fig. 5). Although the in situ methodology in the present study might not show the precise localization because in situ signals were under non-saturated conditions, the BDNF mRNA signals after the mating-induced upregulation were prominent in layer III of the frontal cortex.

Induction of c-*fos* mRNA expression by the mating stimulus was clearly observed in the POA in the vehicle-exposed animals. The patches of in situ positive grains of c-*fos* mRNA were prominent in the POA, including the SDN-POA, at 1 h after mating, whereas the control animals (no mating stimulus) showed no c-*fos* mRNA signals ($P < 0.05$, by Scheffé's post-hoc test, Figs. 4E–G and 5). These results for c-*fos* mRNA localization supported earlier reports showing that the mating stimulus induced *Fos* immunoreactivity in the POA (Robertson et al., 1991; Levine et al., 1995; Coolen et al., 1996; Lumley and Hull, 1999).

Reduction of BDNF mRNA Upregulation by TCDD

The BDNF mRNA upregulation by the mating stimulus was considerably reduced by TCDD (Figs. 4C

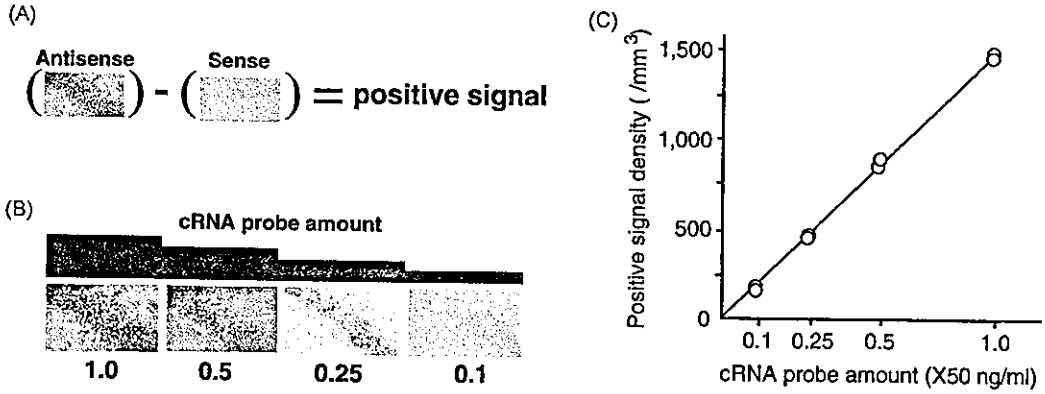

Fig. 3. Semi-quantitative analysis in BDNF mRNA in situ hybridization. (A) The grain density of in situ signals was determined by using the sense probe section as the background, which was mounted on the side of each antisense probe section. (B) Representative video-photographs and (C) linear relationship between the amount of antisense cRNA probe input (5, 25, 50, 100 ng/ml hybridization solution) and the grain density of in situ histochemistry. Lines represent linear regressions for BDNF (Y); $Y = 1.963 \times a$ ($R^2 = 0.999$), where a is the number of cRNA probes for BDNF mRNA. The linear regressions for c-*fos* mRNA was also observed [$Y = 1.960 \times a$ ($R^2 = 0.998$)].

and D and 5). Although BDNF mRNA upregulation was also observed in the 800 ng TCDD/kg-exposed males that showed alteration of sexual behavior, the level of BDNF mRNA expression in the 800 ng TCDD/kg-exposed animals was significantly lower than in the vehicle-exposed males ($P < 0.05$, by Scheffé's post-hoc test). The levels of c-*fos* mRNA expression after the mating stimulus, however, were unaffected by TCDD (Figs. 4G and H and 5). The semi-quantification showed no difference in c-*fos* mRNA expression in the POA between the vehicle- and 800 ng TCDD/kg-exposed groups. These findings imply that TCDD inhibits the neocortical response to sexual behavior.

DISCUSSION

In the present study, perinatal exposure to a low dose of TCDD was found to alter the expression of sexual behavior in the male offspring and to suppress the level of mating-induced BDNF mRNA upregulation, but not to affect the mating-induced c-*fos* mRNA expression or

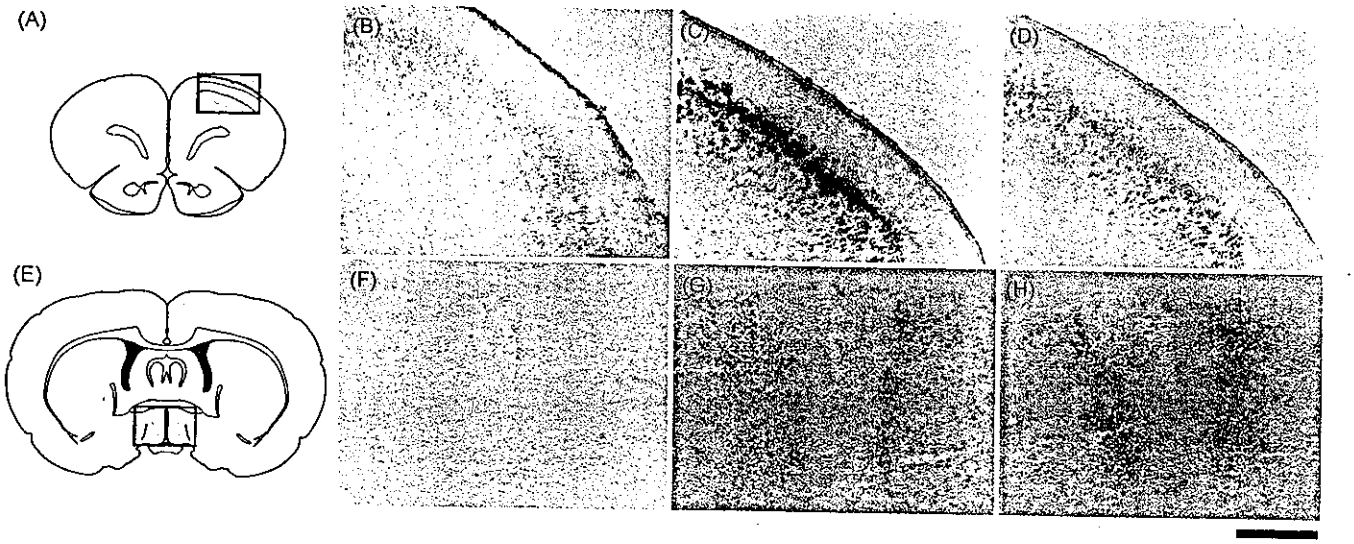

Fig. 4. In situ hybridization of (A–D) BDNF mRNA in the frontal cortex and (E–H) c-*fos* mRNA in the preoptic area, with and without a mating stimulus. (A, E) Brain areas used for the in situ study. Coronal sections modified from the brain atlas (Swanson, 1992). (B) Before the mating stimulus, small BDNF mRNA signals were detected. (C) The mating stimulus induced BDNF mRNA upregulation 1 h after in vehicle-exposed animals. (D) BDNF mRNA expression in 800 ng TCDD/kg-exposed males 1 h after mating. Upregulation was also observed, but the signal density was lower than in the vehicle-exposed animals. (F) c-*fos* mRNA expression was not detected before the mating stimulus in the control animals (0 ng TCDD/kg). (G) The mating stimulus induced c-*fos* mRNA expression in the preoptic area 1 h later in the vehicle-exposed animals. (H) c-*fos* mRNA expression was also observed in the 800 ng TCDD/kg-exposed males. Brain samples were obtained from the CAST + T males that showed alteration of sexual behavior. Scale bar shows 500 μm.

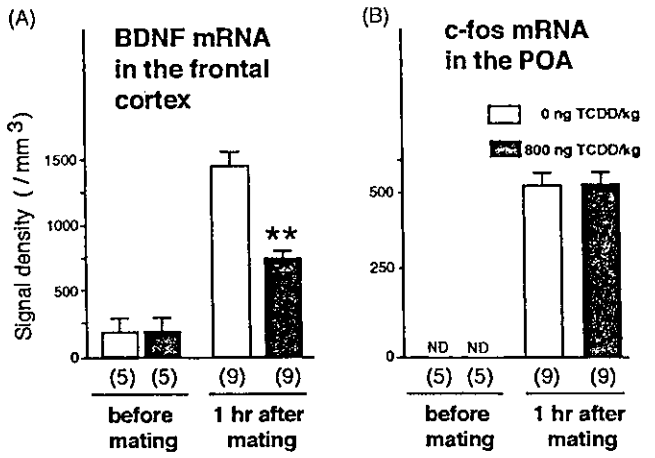

Fig. 5. Semi-quantification of (A) the BDNF mRNA signal in the frontal cortex and (B) the c-*fos* mRNA signal in the POA in vehicle- (open bar) and 800 ng TCDD/kg- (solid bar) exposed groups. Each point is the mean ± S.E. for five (before mating) and nine (1 h after mating) independent samples. The levels of the mating-induced BDNF mRNA upregulation in the 800 ng TCDD/kg-exposed group were significantly lower than in the vehicle-exposed group ($^{**}P < 0.01$, by Scheffé's post-hoc test). There were no significant differences in the levels of the mating-induced c-*fos* mRNA signals between vehicle- and 800 ng TCDD/kg-exposed groups.

the volume of the SDN-POA. These findings indicate that TCDD affected the frontal cortex but not the POA, and altered the expression of sexual behavior.

The BDNF has been identified as a neurotrophic factor and is assumed to participate in activity-dependent synaptic plasticity (Morgan and Curran, 1995). It has been reported that BDNF protein increases in parallel with hippocampal long-term potentiation (Figurov et al., 1996) and that learning tasks induce cortical and hippocampal BDNF mRNA expression in rats (Mizuno et al., 2000) and monkeys (Hashimoto et al., 2000). The present new findings that the mating stimulus upregulated BDNF mRNA expression and that the alteration of sexual behavior was parallel with the level of expression of BDNF mRNA, indicates that BDNF in the frontal cortex, at least in part, participate in the regulation of expression of sexual behavior.

In brain regions that participate in the regulation of male rat sexual behavior, the frontal cortex is assumed to facilitate sexual behavior in association with visual and auditory stimuli. It has been reported that lesions of the frontal cortex inhibited male sexual behavior (Yamanouchi and Arai, 1992) and that tyrosine- and tryptophan-hydroxylase activities in male frontal cortex were increased by the visual stimulus of females (Vega-Matuszczyk et al., 1993). The BDNF mRNA upregulation in the frontal cortex observed in the present study, is thought to be induced by the sexual stimulus of a female and/or sexual intercourse.

Effect of TCDD on expression of sexual behavior has been linked with sexual differentiation of the brain. Mably et al. (1992) has reported that perinatal exposure to TCDD resulted in the inhibition of male sexual behavior, the facilitation of feminine type sexual behavior and the induction of LH surge in the male Holtzman rats. Since male rats, having normal male sexual behavior, also harbor the intrinsic feminine type sexual behavior and/or the LH surge, brain sexual differentiation should be discussed in aspects of 'masculinization', 'de-feminization' and 'feminization'.

In addition, the facilitation of feminine type sexual behavior in male rats is not directly associated with the inhibition of 'de-feminization', since manifestation of feminine type sexual behavior was reported to be inhibited by the septum in the forebrain and the dorsal raphe nucleus in the midbrain, and the destruction of these brain nuclei at the time of adulthood, facilitated feminine sexual behavior in male rats (Kakeyama and Yamanouchi, 1994, 1996). A recent work indicates that perinatal exposure to TCDD reduced the serotonergic immunoreactivities in the raphe nuclei in male mice (Kuchiiwa et al., 2002). This arise the possibility that TCDD affects expression of feminine sexual behavior via the reduction of serotonergic neurons in the dorsal raphe nucleus, independently from de-feminization.

On a topic of 'feminization', induction of LH surge in adult male rats born from TCDD-treated dams seems a sufficient physiological evidence for 'feminization', whereas in a morphological aspect, perinatal exposure to TCDD did not increase the volume of the periventricular area of POA in Holtzman rats (Bjerke et al., 1994). This area is known to be female-dominant in volume, and the increase of its volume is thought to be required for LH secretion (for review, see Parhar and Sakuma, 1997). Further studies are needed to explain the contradiction between the physiological and morphological phenomena.

Regarding 'masculinization', at the start of the present study, we had four working hypotheses on the mechanism of perinatal exposure to dioxin on male sexual behavior (1) inhibition of brain masculinization as a result of disrupting a gonadal steroid during the perinatal period; (2) disruption of testosterone in adulthood; (3) a direct adverse effect on the adult brain; and (4) dysfunction of the adult brain as a consequence of developmental adversity.

The first hypothesis on the 'inhibition of masculinization' was examined by determining the volume of the SDN-POA in male rats that showed behavioral altera-

tions. The volume of the SDN-POA was reported to be 4.5 times larger in males than in females (Gorski et al., 1980), and to depend on the level of perinatal testosterone exposure (Dohler et al., 1982). It is strongly indicated that masculinization does not occur without an increase of the SDN-POA volume. Since the present study failed to show a significant change in the volume of SDN-POA by TCDD exposure, which is consistent with the report by Bjerke et al. (1994), we precluded the first hypothesis.

The second hypothesis, disruption of testosterone in adulthood, is also untenable, for the following reasons. TCDD altered sexual behavior not only in gonadally intact males but also in CAST + T male rats. TCDD reduced the number of mounts and intromissions without changes in the latency of behaviors. It is widely accepted that the latency of behavior increases and the number of behaviors is unaffected or even increases, if the testosterone level decreases or brain masculinization is inhibited (e.g. Kondo, 1992; Robertson et al., 1991; Yamanouchi and Kakeyama, 1992; Vega-Matuszczyk et al., 1993). Gray et al. (1995) has reported that perinatal exposure to TCDD (1 μg/kg on GD15) in Long-Evans rats did not affect androgenic status, while the behavioral change was found in an increase of latency of behaviors, suggesting that the effect remains to be independent of the brain sexual differentiation. Thus, it is indicated that TCDD does not disrupt testosterone at the level which alters expression of sexual behavior.

The third hypothesis, that TCDD has a direct adverse effect on the adult brain, can be ruled out. The GC/MS analysis in the present study showed that brain TCDD levels on PND 120 were reduced to a level close to the detection limit. Such low residual levels of TCDD are too low to directly affect brain function. This third hypothesis is also denied by the results that TCDD did not affect c-*fos* mRNA expression. It has been reported that TCDD can regulate c-*fos* mRNA expression (Puga et al., 1992) through the aryl hydrocarbon receptor and the xenobic recognition element (AhR and XRE) pathway (Fujisawa-Sehara et al., 1988). TCDD is also reported to affect intracellular calcium uptake (Hanneman et al., 1996), while calcium-dependent expressions of c-*fos* and BDNF genes are reported (Morgan and Curran, 1989; Levine et al., 1995; Imamura et al., 2000). Therefore, the residual TCDD concentration in the brain of offspring in adulthood is insufficient to regulate gene expression directly.

Taken together, the fourth hypothesis, that is the dysfunction of the adult brain as a consequence of developmental adversity, seems to be the most plausible. In other word, perinatal exposure to TCDD probably affected neocortical development and as a consequence suppressed the cortical activity-dependent response and the mating-induced BDNF mRNA upregulation.

Our results demonstrate that perinatal exposure to TCDD affects activity-dependent function in the frontal cortex independent of brain sexual differentiation. And yet, no changes in the SDN-POA volume and in c-*fos* mRNA expression in the POA raise the possibility that the POA-hypothalamic axis is not sensitive enough to respond to this dosing regimen of TCDD. Recently, we have reported that perinatal exposure to TCDD altered the levels of NMDA receptor subunit mRNA expression in the neocortex and hippocampus in Long-Evans rats (Kakeyama et al., 2001). Weiss and coworkers reported that prenatal exposure to TCDD at doses lower than our current study, reduced cortical thickness, altered the normal pattern of cortical asymmetry (Zareba et al., 2002), as well as the sexually dimorphic operant behavior (Hojo et al., 2002). It is suggested that the neocortical axis is especially sensitive to TCDD. Further analyses are needed to clarify the effects of TCDD on the neocortical function and the mechanisms affecting the neocortex.

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