

In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin decreases serotonin-immunoreactive neurons in raphe nuclei of male mouse offspring

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Abstract

Female ddY mice were administered 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by gavage for 8 weeks prior to pregnancy. In the male breast-fed offspring born to the TCDD-exposed mice, serotonergic neurons in the brainstem were examined using an immunocytochemical method at 42 days of age. In all offspring, a marked decrease in the intensity of immunostaining occurred in all raphe nuclei compared with the control offspring. The number of serotonin-immunoreactive neurons in each raphe nucleus was measured by computer-assisted analysis. Approximately a quarter to half of immunoreactive neurons were detected in the TCDD-exposed offspring raphe nuclei compared with the control offspring. The present findings suggest that in utero and/or lactational TCDD exposure cause a long-lasting change in the serotonergic system in the raphe nuclei of offspring. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls (PCB; henceforward jointly termed dioxins) are widespread contaminants of the environment and highly toxic tricyclic aromatic compounds [11,14]. This group of compounds is usually considered together because their chemical structures are similar, they share a similar and characteristic pattern of biological responses, and they are believed to have a common mechanism of action [5,13,15]. However, the subgroups vary greatly in potency. The most potent compound of all dioxin congeners is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [15,20].

Dioxins are transferred to each fetus and pup transplacentally and lactationally [8], and dioxins produce a wide variety of toxic effects in offspring. The central nervous system (CNS) during early development may also be a potential target of dioxin [3,6,7,9,10,12,16,19].

Agrawal et al. [1] reported that dopamine levels and dopamine receptor binding sites were decreased in mice prenatally exposed to PCB, and Seegal et al. [17,18] reported that sub-chronic administration of PCB significantly decreased the brain dopamine concentrations in adult male non-human primates, suggesting that dopaminergic neurons are targets of dioxin toxicity. In the present study, we examined serotonin-immunoreactive neurons in the raphe nuclei of male offspring exposed to TCDD in utero and via lactation.

This study was carried out after permission from the Committee of Animal Experimentation, Faculty of Medicine, Kagoshima University. All animals were housed in a Safety Rack (Clea Japan, Inc., Tokyo, Japan) under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$), humidity ($55 \pm 10\%$), and lighting (lights on at 07:00 h, off at 19:00 h). Food and water were administered ad libitum.

TCDD (99.1% purity) was purchased from Accu Standard, Inc. (New Haven, CT). A total of 21 female adult ddY mice were administered an oral dose of TCDD (A-group, 4.9×10^3 pg/kg, $n = 7$; or B-group, 4.9×10^5 pg/kg, $n = 7$) or an equivalent volume of vehicle (olive oil, 6.7 ml/kg, $n = 7$) by gavage. The dose administration was

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carried out once a week for 8 weeks (total of 3.92×10^4 or 3.92×10^6 pg/kg, respectively). Immediately after the last administration, the mice were housed together with normal male mice for mating. Nine TCDD-exposed female mice (three mice per group) bore 10–12 offspring at 20 or 21 days after mating. One day after birth, in these cases, each litter was adjusted to ten infants to allow for similar lactational TCDD exposure. The offspring were weaned at postnatal day 28 and group-housed in plastic cages. Three male TCDD-exposed offspring and control offspring per litter (total of 27 offspring; body weight, 30–32 g) were used for an immunocytochemical study at 42 days of age.

The offspring were deeply anesthetized with intraperitoneal injection of sodium pentobarbital (100–200 ml/kg), and each offspring was perfused with 20 ml of 0.1 M sodium phosphate-buffered saline (pH 7.4), followed by 200 ml of freshly prepared 4% buffered paraformaldehyde containing 0.2% picric acid at 4°C. The brain was cut stereotactically in the coronal plane, removed and immersed in fixative for an additional 2 h and transferred to 30% sucrose in phosphate buffer (pH 7.4) at 4°C. Serial 40- μ m-thick coronal sections were cut on a freezing microtome. Every second serial section of the brainstem was processed for immunocytochemistry. Adjacent sections were counterstained with thionin after the immunocytochemical procedure to facilitate subsequent anatomical localization and cytoarchitectural analysis.

The immunocytochemical procedures were performed on free-floating sections. The sections were preincubated in a solution of 0.3% Triton X-100, 1% goat serum and 0.3% bovine serum albumin in phosphate-buffered saline (PBS) after blocking endogenous peroxidase, and they were then incubated with antibodies to serotonin developed in rabbits (DiaSorin, Stillwater, MN) diluted 1:10,000 in PBS (pH 7.4) containing 0.3% Triton X-100 at 4°C for 72 h. The sections were rinsed and incubated in a 1:2000 dilution of goat anti-rabbit IgG (EY-Laboratories, San Mateo, CA) for 24 h at 4°C and rinsed again thoroughly with PBS. The sections were treated in a 1:2000 dilution of rabbit peroxidase-anti-peroxidase (DAKO A/S, Glostrup, Denmark) for 24 h at 4°C, washed with PBS, and treated for 9 min at room temperature with 0.02% diaminobenzidine in 0.001% hydrogen peroxide. The antibodies were diluted to appropriate concentrations in 0.3% bovine serum albumin and 1% normal goat serum. In each case, the immunocytochemical procedure was performed under the same conditions. The specificity of the immunocytochemistry for serotonin was tested by substitution of normal rabbit serum for the purified rabbit antibody to serotonin or by omitting the primary antibody to serotonin from the incubating medium.

A total of 18 offspring (six male mice per group) were used in the cell count analysis. The cell counts were carried out in the dorsal raphe nucleus, the median raphe nucleus, the suprallemniscal area (B9) and the Nucleus raphe magnus. Three sections through each nucleus (the same levels in each offspring) were photomicrographed at $\times 50$ magnifica-

tion under the same conditions, and then each 35 mm photographic positive film was scanned at a resolution of 3000 dpi using a Nikon Film scanner LS-4500. Immunoreactive neurons in each bilateral nucleus were counted blindly with regard to treatment using the public domain NIH Image program. The criteria for counting a neuron as immunopositive for serotonin were as follows: first, the neuron was stained more intensely than the highest level of background; and second, the neuron possessed over 300 pixels as a valid stained area. For statistical analysis of the differences between TCDD-exposed offspring and control mice, a one-way analysis of variance (ANOVA) followed by Dunnett's method was used. Significance was set at $P < 0.01$.

The staining in our material was specific, as is shown by the following arguments. First, when the primary antibody to serotonin was replaced with the normal serum or when the primary antibody was omitted, no reaction could be observed in any of the nuclei strongly positive with the specific antibody. Second, the brainstem areas containing high numbers of dopaminergic (substantia nigra) and catecholaminergic (locus coeruleus) neurons were not stained, although serotonergic neurons in the same section revealed intense staining, reflecting a lack of cross-reactivity between serotonin and the other monoamines in the brainstem. Third, staining with another primary antibody (Sigma, St. Louis, MO) resulted in the same pattern of staining, and the labeled neurons were restricted to the raphe system.

Following immunocytochemical staining of serial sections cut from the entire length of the brainstems in 42-day-old control offspring, serotonin-immunoreactive neurons were distributed throughout the entire brainstem. The staining properties and the distributions of the immunoreactive neurons were similar in all male control offspring. Immunocytochemically stained serotonergic neurons were distributed in the caudal linear nucleus, the median raphe nucleus, the dorsal raphe nucleus (Fig. 1A), the Nucleus raphe pontis, the interpeduncular nucleus, the suprallemniscal area, the pedunculopontine tegmental nuclei, the deep mesencephalic nucleus, the Nucleus raphe magnus (Fig. 1C), the Nucleus raphe pallidus, the Nucleus raphe obscurus, dorsal and medial to the facial nucleus and the ventrolateral medulla.

In the male TCDD-exposed offspring, the labeled neurons were similarly distributed in the raphe nuclei. However, a marked decrease in intensity of immunostaining occurred in all raphe nuclei, and consequently, the detectable serotonin-immunoreactive neurons were greatly decreased in number. In the raphe nuclei of the B-group offspring, the immunostaining reaction product was observed to be slightly lighter than that in the A-group under a light microscope. Fig. 1B,D shows the staining properties of serotonergic neurons in the raphe nuclei of the TCDD-exposed offspring.

The decreases in staining intensity occurred almost uniformly in each raphe subnucleus. In the computer-assisted cell counting in a total of 12 sections cut from one control mouse, an average of 1573.5 immunoreactive

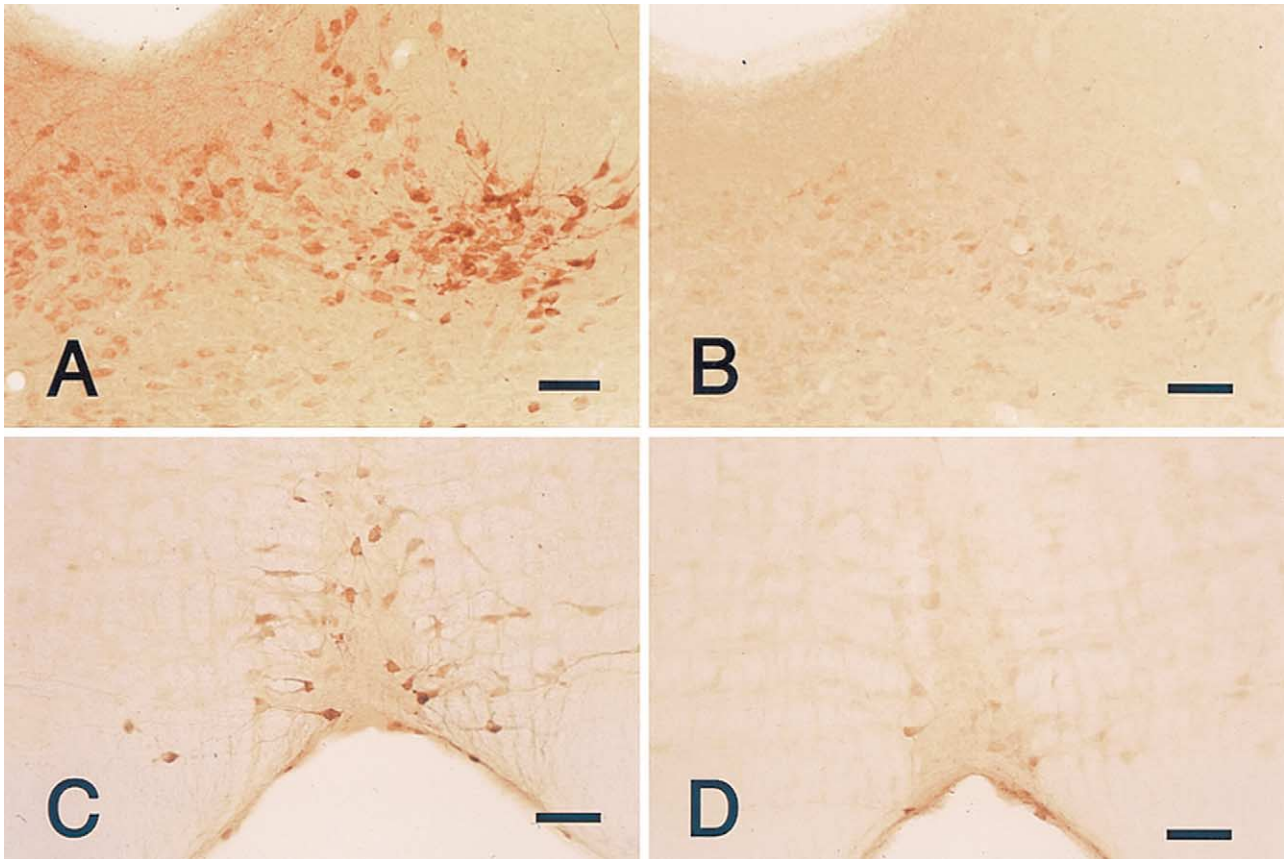


Fig. 1. Photomicrographs of coronal sections through the dorsal raphe nucleus (A,B) and the Nucleus raphe magnus (C,D) processed for serotonin immunocytochemistry. (A,C) Sections from the control offspring; (B,D), sections from the TCDD-exposed offspring (B, B-group; D, A-group). Scale bars, 50 μm .

neurons were detected ($n = 6$). In the A- ($n = 6$) and B-groups ($n = 6$), however, only 716.3 and 419.8 neurons (about 45.5 and 26.7% compared with the control group) were counted, respectively (Fig. 2). The numbers of immunoreactive neurons in the raphe nuclei of both groups of TCDD-exposed offspring were significantly lower than in those of the control mice ($P < 0.01$).

The present findings suggest that in utero and/or lactational exposure to TCDD is responsible for the marked decrease of detectable serotonin-immunoreactive neurons in the raphe nuclei of male offspring, suggesting that TCDD may act as a neuroteratogen, producing long-term alterations in neuronal serotonin synthesis and serotonergic function.

In the present study, no essential differences between the male TCDD-exposed offspring and the control male offspring were noted in external morphology, birth and postnatal growth weight or nutrition. However, the TCDD-exposed offspring showed tendencies to be aggressive against other normal mice and to be hypersensitive to soft touch. For example, when the TCDD-exposed offspring was put into a small cage together with a normal mouse, the TCDD-exposed offspring regularly opened an attack against the normal mouse. When the TCDD-exposed offspring was

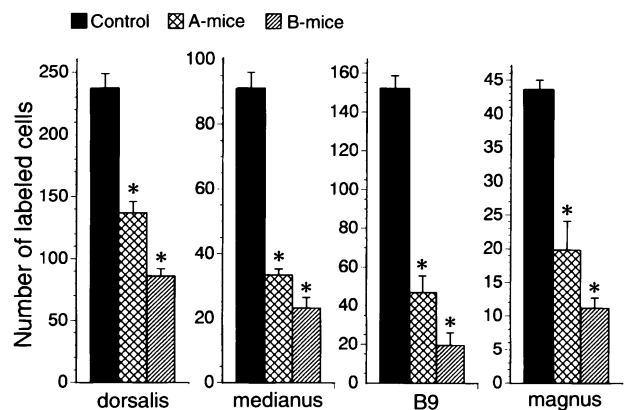


Fig. 2. Quantitative evaluation of serotonin-immunoreactive neurons in each nucleus of the controls (black columns), A-group (cross-hatched columns) and B-group offspring (hatched columns), respectively. Immunoreactive neurons were counted in the dorsal raphe nucleus (dorsalis), the median raphe nucleus (medianus), the supramedianal area (B9) and the Nucleus raphe magnus (magnus). The height of each column indicates the mean of the number of immunoreactive neurons in one section, and the associated vertical line is the SEM ($n = 6$). Asterisks indicate significant differences between control and TCDD-exposed offspring, determined by a one-way ANOVA followed by Dunnett's test ($P < 0.01$).

touched on the side of the chest with a stick, the animal brushed it away with its hind leg. Such actions of attack and hypersensitivity were not observed in the control mice. These behavioral changes in in utero and lactational TCDD-exposed offspring may be caused by the alterations in the neurochemical mechanisms.

It has been known that in utero and lactational TCDD exposure increase the expression of lordotic behavior in male offspring [2]. Since this phenomenon is not associated with alterations in estrogen receptor binding or volumes of sexually differentiated brain nuclei of the hypothalamus, and since decreasing serotonin and dopamine levels in the CNS exert an influence on sex behavior in males [2], it is assumed that such a phenomenon of feminization of sex behavior was produced by decreases in both the serotonin and dopamine levels in the CNS.

Prenatal and early postnatal exposure to PCBs resulted in delays in psychomotor development and cognitive function in children [4,6,7], and long-term changes in behavior in laboratory rodents and non-human primates [9,19]. In the course of the present study, we observed behavioral changes in TCDD-exposed offspring. It is possible that in utero and lactational exposure to dioxins causes serious long-lasting behavioral, mental and psychic impairments in offspring. We have not been able to clarify whether either in utero or lactational exposure to dioxins, or both, are causative factors in the neurochemical impairments.

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