Bcl-2 Gene Family Expression in the Brain of Rat Offspring after Gestational and Lactational Dioxin Exposure

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ABSTRACT: Recent epidemiological studies have shown that dioxin, a persistent organic pollutant, is related to cognitive and behavioral abnormalities in the offspring of exposed cohort. In order to investigate the possible impact of dioxin in survival gene expression during brain development, we established an animal model of gestational and lactational dioxin-exposed rat offspring. The expressions of dioxin-responsive gene cytochrome P450 1A1 (CYP1A1), apoptotic gene Bax, and anti-apoptotic genes Bcl-2 and Bcl-x_L were examined in rat liver and brains using Western blot analysis and RT-PCR. The results showed that treatment of pregnant rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (2 μg/kg body weight through oral delivery) at gestation day 15 resulted in an increase of Bcl-x_L in offspring male liver and cerebral cortex, but a decrease in female offspring. In contrast, the expression of Bcl-x_L in the cerebellum was decreased in male, but increased in female. Bcl-2, another anti-apoptotic gene, was also downregulated in P0 female liver, cerebral cortex, but was not observed in male. In the 4-month-old offspring, however, the Bcl-2 protein levels in the liver and cerebellum of both male and female pups were higher in the TCDD group as compared with the control group. However, the Bcl-2 level in the cerebral cortex of TCDD-treated groups was higher than the control group only in female but not male offspring at 4 months old. The expression of Bax showed no significant changes upon TCDD exposure at P0 stage, but was significantly reduced in the 4-month-old male cortex. These results indicate that early exposure of dioxin could affect the development of certain brain regions with gender difference, in terms of its differential effect on expressions of Bcl-x_L, Bcl-2, and Bax.

KEYWORDS: dioxin; brain; Bcl-2; Bcl-x_L; Bax; cerebral cortex; cerebellum; liver

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INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental pollutant and a toxic member of a family of compounds known as "endocrine-disrupting chemicals" (EDCs). Maternal TCDD exposure can lead to early pre- and postimplantation embryo loss.[1] TCDD-related toxicity is mainly mediated by the aryl hydrocarbon receptor (AhR) protein, which binds TCDD and heterodimerizes with its partner protein, the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR–ARNT complex binds to dioxin-responsive elements (DREs) and enhances the transcription of the phase I drug-metabolizing (*cytochrome P4501A1)* gene and phase II (*UDP-glucronosyltransferase*, *glutathione S-transferase)* gene.[2] As such, the function of the CYP1A1 product is metabolic activation. However, TCDD undergoes oxygenation by the CYP1A1 product, and ultimately leads to be the highly mutagenic derivatives. An alternative mechanism of CYP1A1 activation leads to oxidative stress by the production of H_2O_2.[3,4] Oxidative stress is known to cause DNA damage and P53-dependent apoptosis.[5] Oxidative stress and inappropriate apoptosis may be involved in abnormal neuron development and neurodegeneration. Recent epidemiological studies have shown that dioxin exposure is related to cognitive and behavioral abnormalities in the offspring of exposed cohort.[6,7] In this study, we established an animal model to demonstrate that early exposure of dioxin could affect the development of certain brain regions in a gender-different manner in terms of its differential effect on Bcl-2 gene family expressions. Apoptotic gene expressions, such as anti-apoptotic–promoting protein Bcl-2 and Bcl-x_L and pro-apoptotic protein Bax in the offspring of TCDD-treated rat brain tissues were examined.

MATERIALS AND METHODS

Animal Model of Gestational and Lactational Exposure of TCDD in Rats

Pregnant Sprague-Dawley (SD) rats were received from the National Institute of Experimental Animal Research, Taipei, Taiwan, on day 13 of gestation (GD13). TCDD was orally administered to the maternal rats on GD15 by gavage with a single dose of TCDD (2 μg/kg/2 mL corn oil) or vehicle (corn oil 2 mL/kg). Liver, cerebral cortex, and cerebellum were collected from offspring of TCDD-treated (T) or corn oil-treated (C) SD rats on the day of birth (P0) and at 4 months of age (115–125 days) for Western blot and RT-PCR analysis. The protocol of this animal treatment was reviewed by the Experimental Animal Committee at Taipei Medical University. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Western Blot Analysis

Tissues collected were homogenized with ice-cold homogenizing medium (0.32 M sucrose, and 50 mM Tris-HCl containing 1mM EDTA, 1mM sodium orthovanadate, 1 mM PMSF 10 μg/mL trypsin/chymotrypsin inhibitor, 1 mM bezamidine, and 0.1 mM leupeptin, pH 7.4). Each sample protein (30–90 μg) was separated onto 7.5% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed with proper diluted specific

primary antibody. The immune complex was further probed with HRP-conjugated goat anti-rabbit IgG, visualized by HRP-reactive chemiluminescence reagents (Pierce, Rockford, IL), and developed on autoradiographic film (Kodak BioMax film, Eastman Kodak Co., Rochester, NY). The relative density of the protein band in the Western blot was further analyzed with an electrophoresis image analysis system (Eastman Kodak Co.). Net intensity of each protein band was normalized with the intensity of housekeeping GAPDH protein, and was then divided by the normalized intensity of the control group to obtain the ratio-to-control value. Data was further analyzed by unpaired *t*-test, and the graph was plotted using SigmaPlot 2001 (SPSS Inc., Chicago, IL).

Reverse Transcribed–Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from the liquid nitrogen quick-frozen tissues by directly homogenizing the tissues in extraction buffer (Trizol/phenol/chloroform), and mRNAs were reversed transcribed into cDNA using oligo-dT and reverse transcriptase (Invitrogen). Primer sets of rat β-actin or GAPDH genes and 2.5 μg of total RNA extracted from liver, cortex, or cerebellum were used. The level of housekeeping genes β-actin and GAPDH expressions were analyzed and used to demonstrate the presence of the same amount of total cDNA in each RNA sample. The expression levels of Bcl-2, Bcl-x_L, Bax, and internal control β-actin and GAPDH were detected from the same samples using designed primers. Sequences of the primer pair for amplification of each gene were 5′-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3′ and 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ (for β-actin gene); 5′-GAC CCC TTC ATT GAC CTC AAC-3′and 5′-GAT GAC CTT GCC CAC AGC CTT-3′ (for glyceraldehydes-3-phosphate dehydrogenase; GAPDH gene); 5′-CAC CAG CTC TGA ACA GYY CAT GA-3′ and 5′-TCA GCC CAT CTT CTT CCA GAT GGT-3′ (for Bax gene); 5′-CAC CCC TGG CAT CTT CTC CTT-3′ and 5′- AGC GTC TTC AGA GAC AGC CAG-3′ (for Bcl-2 gene); 5′-AGGCTG GCGATG AGTTTG AA-3′ and 5′-TGA AAC GCT CCT GGC CTT TC-3′ (for Bcl-x_L gene). All amplification primers were synthesized bt GIBCO-BRL Technology. PCRs included 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55–60°C (various for each primer set) and extending for 1 min at 72°C. Reaction products were separated on 2% agarose gel. Band density was quantitated using Image analysis software as described in the Western blot analysis section, MATERIALS AND METHODS.

RESULTS AND DISCUSSION

Bcl-2 Gene Family Expression in the Liver of Gestational and Lactational Dioxin-Exposed Rat Offspring

Since liver is the primary target of dioxin, we first examined the expression profile of the Bcl-2 gene family in the liver of P0 pups and 4-month-old offspring. FIGURE 1 shows that the primary responsive gene CYP1A1 was strongly induced in the liver of both male and female P0 pups, assuring the positive response of the offspring rats to TCDD. The protein levels of CYP1A1 in TCDD-exposed male offspring at

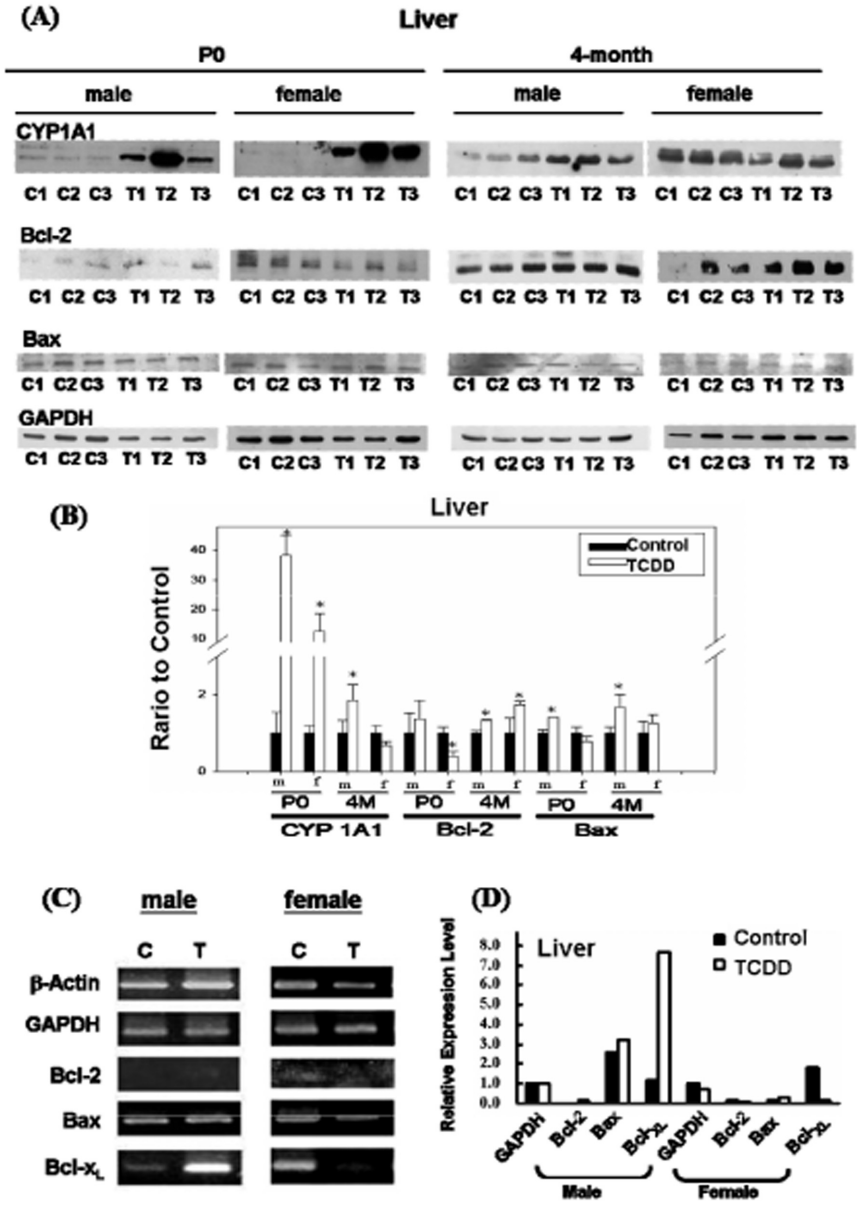

FIGURE 1. Western blot and RT-PCR analysis of expressions of CYP1A1, Bcl-2, Bcl-x_L, and Bax genes in liver of gestational and lactational dioxin-exposed rat offspring. Pregnant SD rats were orally administered with a single dose of TCDD (2 μg/kg/2 mL) or vehicle (corn oil 2 mL/2 kg) on GD15. All lobes of the liver were collected from offspring of TCDD-treated (T) or corn oil–treated (C) rats at P0 and at 4 months of age for Western blot (**A** and **B**) and RT-PCR analysis (**C** and **D**). **A** and **B**: The protein levels of CYP1A1, Bcl-2, and Bax

4 months were still higher than the control group. However, there were no significant changes of the CYP1A1 protein levels observed in 4-month-old female offspring, indicating that the primary effect of TCDD was only observed in the newborn stage, but did not sustain to the adult stage. These observations suggest that CYP1A1 may function as an acute factor for the TCDD-elicited toxicity in the newborn stage. Subsequent signals triggered by long-term exposure of TCDD, such as the desensitization of AhR, might also participate in TCDD neurotoxicity.

Examination of the Bcl-2 gene family expression showed that Bcl-x_L was much higher in the P0 TCDD-exposed male liver, but much lower in the P0 TCDD-exposed female liver as compared with their respective controls. The expression of Bcl-2 does not show not much difference between the control and the TCDD groups of P0 male, but seems to be lower in the P0 female of the TCDD group. Interestingly, TCDD-exposed offspring at 4 months had higher Bcl-2 expression in liver of both male and female offspring. The expression of pro-apoptotic gene Bax in liver was not significantly affected by the TCDD exposure in the age and gender of the offspring investigated. These results suggest that gestational exposure to dioxin, as shown in the responses of P0 pups, may induce a protective signaling in male livers, but not in female, in terms of the profound increased of Bcl-x_L expression. On the other hand, livers of female offspring might be more susceptible to dioxin cytotoxicity because both Bcl-2 and Bcl-x_L were profoundly downregulated. The higher expression of Bcl-2 in the liver of the TCDD-exposed 4-month-old female offspring suggests a possibility of carcinogenesis as a long-term effect, as reported in other literature on dioxin-induced carcinogenesis in liver.[7,8] Another possibility is that neonatal exposure to TCDD may cause a pre-stress to the liver, which in turn induces a self-protective mechanism, such as the induction of Bcl-2 expression for survival of the tissue.

Bcl-2 Gene Family Expression in the Cerebral Cortex of Gestational and Lactational Dioxin-Exposed Rat Offspring

The cerebral cortex controls the cognitive function that has been reported to be impaired in TCDD-exposed offspring in human. We, therefore, examined the expression profile of the Bcl-2 gene family in the cerebral cortex of P0 pups and 4-month-old offspring. FIGURE 2 shows that the primary responsive gene CYP1A1 was not greatly induced in the cerebral cortex of both male and female offspring at either P0 or 4 months old. However, in another study of our lab using primary cultured cortical neurons, we showed that TCDD could indeed induce CYP1A1 expression in a transient manner (unpublished data). Therefore, it is possible that the response of cerebral cortex to TCDD is rather transient as compared with the response in the liver.

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in both P0 and 4-month-old male (m) and female (f) offspring upon TCDD exposure are shown. Data were calculated as described in MATERIALS AND METHODS, and were expressed as ratio to control (mean ± SEM, $n = 4$). $^*P < 0.05$, as compared with their respective controls. **C** and **D**: Expression levels of Bcl-2, and Bax, and Bcl-x_L in P0 male and female offspring upon TCDD exposure were analyzed by RT-PCR. For quantitation, the levels both of protein amount and gene expression were normalized with the level of housekeeping GAPDH gene expression.

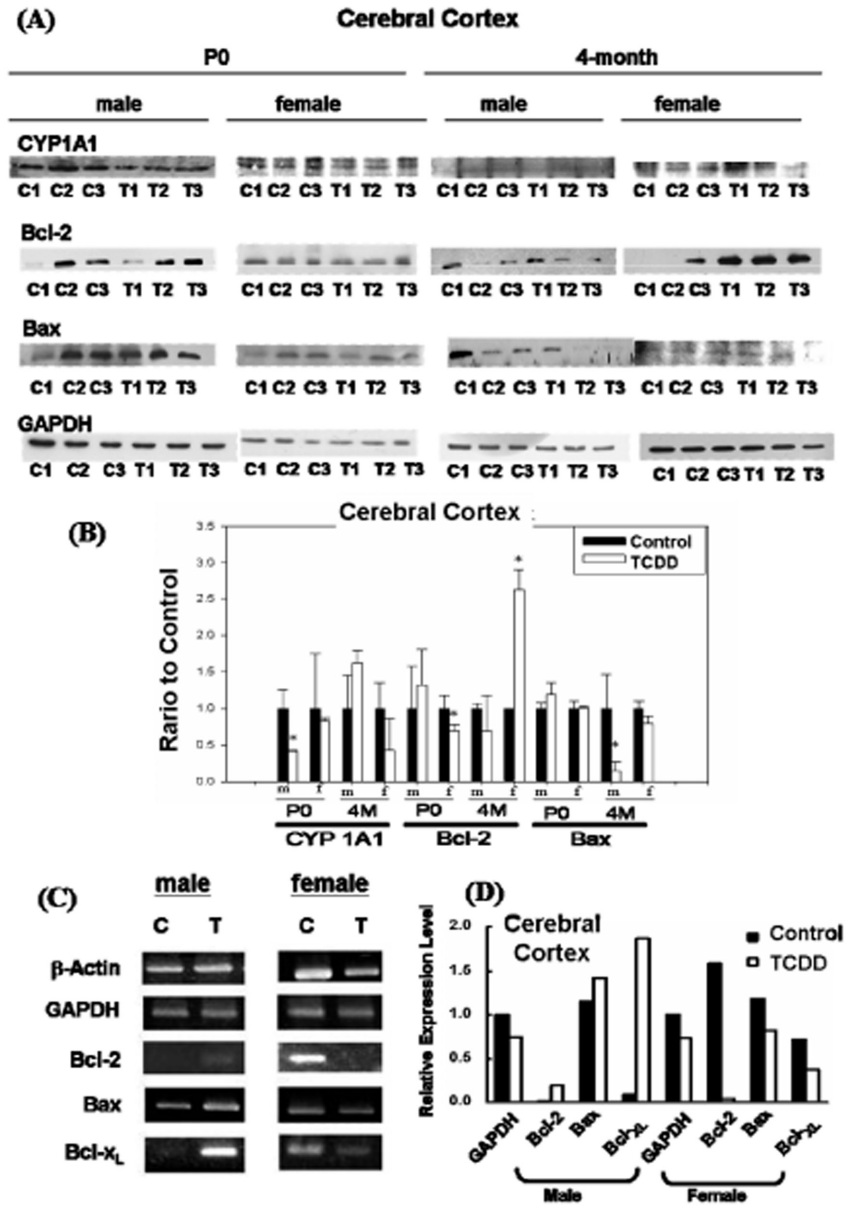

FIGURE 2. Western blot and RT-PCR analysis of expression of CYP1A1, Bcl-2, Bcl-x_L, and Bax genes in cerebral cortex of gestational and lactational dioxin-exposed rat offspring. Pregnant SD rats were orally administered with a single dose of TCDD (2 μg/kg/2 mL) or vehicle (corn oil 2 mL/2 kg) on GD15. Cerebral cortex was collected from offspring of TCDD-treated (T) or corn oil–treated (C) rats at P0 and at the 4 months of age for Western

Alternatively, it could reflect the fact that cerebral cortex is less responsive to TCDD in terms of the induction of CYP1A1 gene expression. There are several reports showing that dioxin or other PCBs have non-AhR-mediated effect in central neurons,[9] which subsequently results in a low induction level of CYP1A1.

In terms of the Bcl-2 gene family expression, expression profiles of both Bcl-2 and Bcl-x_L genes in the P0 pup cortex were similar to that in the P0 pup liver. Bcl-x_L was much higher in the P0 TCDD-exposed male cortex, but much lower in the P0 TCDD-exposed female cortex as compared with their respective controls. The expression of Bcl-2 was not much different in P0 male cortex, but was lower in the P0 female of the TCDD group. Similar to its expression profile in liver, the Bcl-2 expression of the TCDD group of 4-month-old female cerebral cortex was also much higher than the control group, but not in 4-month-old male offspring. These results suggest that cerebral cortex of female offspring at neonate stage might be more susceptible to the dioxin cytotoxicity since both Bcl-2 and Bcl-x_L were profoundly downregulated at P0. In addition, the expression of Bax in cerebral cortex was significantly reduced by the TCDD exposure in 4-month-old male but not female offspring. These results suggest that gestational exposure to dioxin may induce differential protective machinery in male and female cortex, with decreased Bax and increased Bcl-2 expression, respectively. Moreover, this observation raised the possibility that cerebral cortex of female rats might develop a long-term protective mechanism due to TCDD exposure in the fetal stage. This possibility comes from the fact that in our animal model system, TCDD-exposed offspring had a much higher mortality rate than the vehicle-exposed offspring (unpublished observation). Since the 4-month-old TCDD-exposed offspring rats were the "survivors" of the toxin insult, it is likely that they survived by means of upregulating protective machineries, such as Bcl-2 against apoptosis. These results also imply that there might be gender difference in the cognitive deficit observed in offspring of cohort upon gestation and lactation exposure to dioxin.

Bcl-2 Gene Family Expression in the Cerebellum of Gestational and Lactational Dioxin-Exposed Rat Offspring

The cerebellum serves functions related to working memory and motor planning, which also has been reported to be impaired in TCDD-exposed offspring in human. Accordingly, we examined the expression profile of the Bcl-2 gene family in the cerebellum of P0 pups and 4-month-old offspring. FIGURE 3 shows that the primary responsive gene CYP1A1 was not induced much in the cerebellum of both male and female offspring either at P0 or 4 months old. This result was similar to the one ob-

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blot (**A** and **B**) and RT-PCR analysis (**C** and **D**). **A** and **B**: The protein levels of CYP1A1, Bcl-2, and Bax both in P0 and 4-month-old male (m) and female (f) offspring upon TCDD exposure are shown. Data were calculated as described in MATERIALS AND METHODS, and were expressed as ratio to control (mean ± SEM, $n = 4$). $^*P < 0.05$, as compared with their respective controls. **C** and **D**: Expression levels of Bcl-2, and Bax, and Bcl-x_L in P0 male and female offspring upon TCDD exposure were analyzed by RT-PCR. For quantitation, the levels both of protein amount and gene expression were normalized with the level of housekeeping GAPDH gene expression.

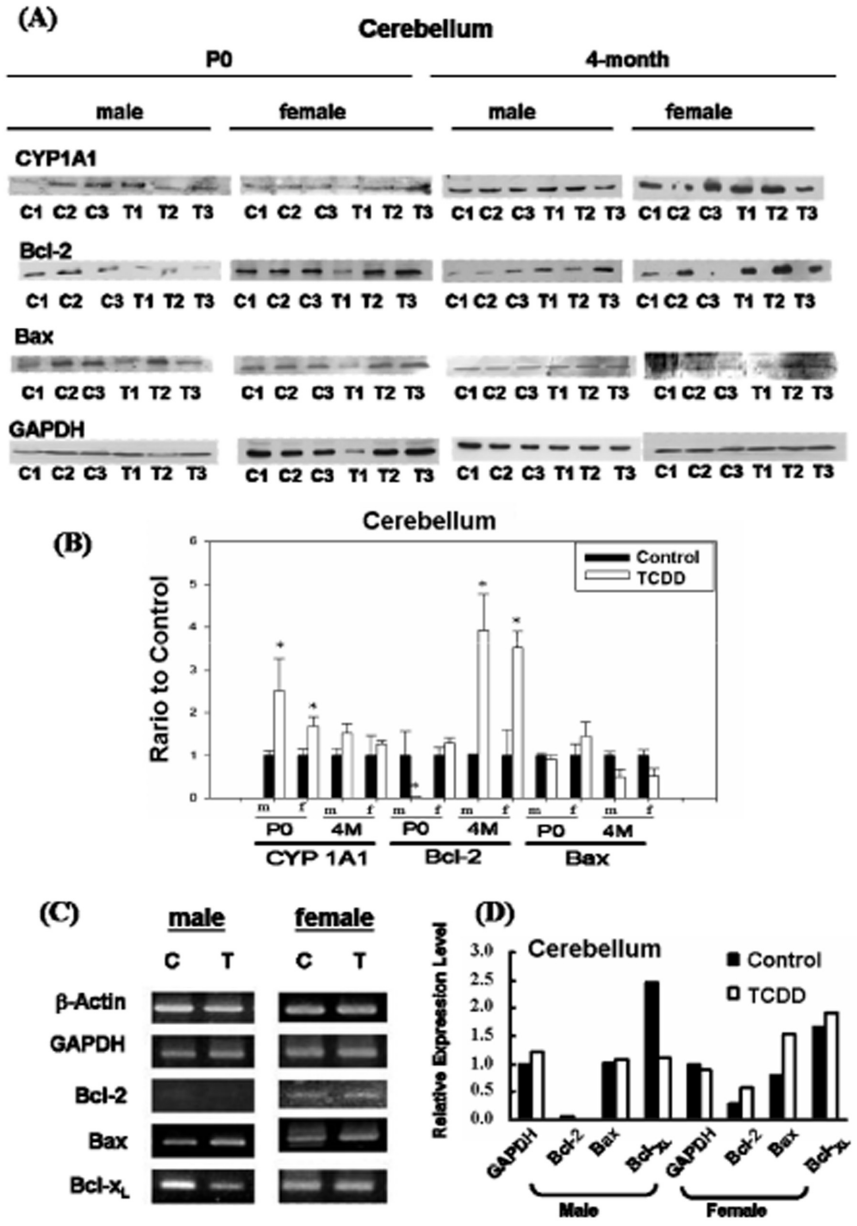

FIGURE 3. Western blot and RT-PCR analysis of expressions of CYP1A1, Bcl-2, Bcl-x_L, and Bax genes in the cerebellum of gestational and lactational dioxin-exposed rat offspring. Pregnant SD rats were orally administered with a single dose of TCDD (2 μg/kg/2 mL) or vehicle (corn oil 2 mL/2 kg) on GD15. Cerebellum was collected from offspring of TCDD-treated (T) or corn oil–treated (C) rats at P0 and at 4 months of age for Western blot

tained in the cerebral cortex, again implicating that the primary gene induction of TCDD in the brain might be transient in general as compared with the persistent elevation in liver.

The Bcl-2 gene family expression of the cerebellum of TCDD-exposed offspring has a distinct pattern different from those in liver and cerebral cortex. Bcl-x_L was much lower in the P0 TCDD-exposed male cerebellum, but no significant difference was observed in the P0 TCDD-exposed female cerebellum as compared with their respective controls. Bcl-2 was significantly lower in the P0 male cerebellum of the TCDD group, but was lower in the P0 female of the TCDD group. Similar to the expression profile in liver, the Bcl-2 expression in the cerebellum of the TCDD group was higher than the control group of both male and female offspring at 4 months. The expression of Bax in cerebellum was not significantly affected by the TCDD exposure in the age and gender of the offspring investigated. These results suggest that gestational exposure to dioxin induced a downregulation of both Bcl-2 and Bcl-x_L gene expressions in the cerebellum of male but not female offspring, rather than upregulation observed in the cerebral cortex. On the other hand, cerebellum of the female offspring might not be as susceptible as the liver and cerebral cortex to the dioxin cytotoxicity because both Bcl-2 and Bcl-x_L are not downregulated much. Higher expression of Bcl-2 was also observed in the cerebellum of both male and female TCDD-exposed offspring at 4 months. Similar explanations to the ones proposed for the Bcl-2 upregulation in the cerebral cortex are under investigation. Moreover, these results imply that the anti-apoptotic genes in different brain region are differentially regulated by dioxin.

CONCLUSION

The information obtained from this study suggests that the male and female offspring rats respond to the gestational dioxin exposure differently. The impact of the profound alteration of Bcl-x_L, Bcl-2, and Bax expressions in cerebral cortex and cerebellum of the dioxin-exposed offspring sheds a new light on the study of tissue-specific, brain region–specific, and gender-dependent neurodegeneration, possibly derived from environmental pollution. The differences may be due to the differential distribution and expression level of the Ah receptor in individual tissue, as well as the dioxin-elicited differential interruption of sex hormone functions in male and female.

(**A** and **B**) and RT-PCR analysis (**C** and **D**). **A** and **B**: The protein levels of CYP1A1, Bcl-2, and Bax both in P0 and 4-month-old male (m) and female (f) offspring upon TCDD exposure are shown. Data were calculated as described in MATERIALS AND METHODS , and were expressed as ratio to control (mean ± SEM, $n = 4$). $^*P < 0.05$, as compared with their respective controls. **C** and **D**: Expression levels of Bcl-2, and Bax, and Bcl-x_L in P0 male and female offspring upon TCDD exposure were analyzed by RT-PCR. For quantitation, the levels of both protein amount and gene expression were normalized with the level of housekeeping GAPDH gene expression.

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