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# **AhR-Mediated Effects of Dioxin on Neuronal Acetylcholinesterase Expression *in Vitro***

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**Running Title:** Transcriptional regulation of neuronal AChE by dioxin

**Key words:** Acetylcholinesterase (AChE), aryl hydrocarbon receptor (AhR), dioxin responsive  
element (DRE), neuron, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), transcriptional regulation

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**Abbreviations:** AChE, acetylcholinesterase; AChE<sub>R</sub>, AChE R subunit; AChE<sub>T</sub>, AChE T subunit; -

AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DLCs, -

dioxin-like compounds; DRE, dioxin responsive element; PCBs, polychlorinated biphenyls; -

PCDDs, polychlorinated dibenzo-p-dioxins; PCDFs, polychlorinated dibenzofurans; PeCDF, -

2,3,4,7,8-pentachloro-dibenzofuran; PRiMA, proline-rich membrane anchor; TCDD, 2,3,7,8-

tetrachlorodibenzo-p-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran. -

## Abstract

**BACKGROUND:** Deficits in cognitive functioning can be found in victims exposed to dioxins and dioxin-like compounds. Evidence suggests that dioxins induce cholinergic dysfunction mediated by hypothyroidism. However, little is known about direct effects of dioxins on the cholinergic system.

**OBJECTIVES:** We investigated the action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on acetylcholinesterase (AChE), a key enzyme in cholinergic neurotransmission.

**METHODS:** Cultured human-derived neuronal cells were employed to evaluate the effect of exposure to dioxin on AChE.

**RESULTS:** A significant decrease in enzymatic activity of AChE was found consistently in cultured neurons treated with TCDD. We also found that, unlike organophosphate pesticides that directly act on the catalytic center of AChE, the suppressive effect of dioxin was through transcriptional regulation. Addition of an inhibitor of the aryl hydrocarbon receptor (AhR)-dependent pathway counteracted the TCDD-induced suppression of AChE, suggesting an involvement of the AhR-dependent pathway. The existence of putative dioxin-responsive element (DRE) consensus sequences in the human *ACHE* promoter region further supported this hypothesis. Consistent with the absence of DRE elements in mouse or rat *ACHE* promoter regions, suppression of AChE by TCDD did not occur in rat neuronal cells, indicating a potential species-specific effect.

CONCLUSIONS: We demonstrated that dioxin suppressed the activity of neuronal AChE via AhR-mediated transcriptional down-regulation. This is the first study to report direct interference by dioxin with the cholinergic neurotransmission system.

## Introduction

The polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs) and related dioxin-like compounds (DLCs) represent a diverse group of contaminants, many of which are highly toxic and both environmentally and biologically persistent (reviewed in Mandal 2005). Dioxins or DLCs cause multiple toxic effects, including increased risk of cancer and interference with the function and development of the nervous, immune and reproductive systems (Boucher et al. 2009; reviewed in Marinković et al. 2010; reviewed in White and Birnbaum 2009).

Cholinergic neurotransmission and acetylcholinesterase (AChE), a vital functional enzyme in cholinergic neurotransmission, play important roles in multiple advanced brain functions, such as memory, learning and attention (Hasselmo and Sarter 2011; reviewed in Soreq and Seidman 2001; reviewed in Woolf and Butcher 2011). Emerging evidence suggests that maternal or perinatal exposure to dioxins or DLCs can interfere with the development of the central cholinergic system, including the development of AChE in the cerebellum (Ahmed 2011) and expression of muscarinic acetylcholine receptors in the cerebrum and cerebellum of rats (Coccini et al. 2007). Ahmed (2011) suggested that the effects of TCDD on brain AChE were related to alterations in thyroid development. It is also plausible that dioxins could directly affect cholinergic neurotransmission, a possibility that we explored here.

In general, activity of AChE can be affected in two ways: direct inhibition of enzymatic activity or suppression of transcription. Inhibition of AChE activity has been used as an indicator

of organophosphorus insecticide (OP) exposure, because OPs irreversibly inhibit the activity of AChE by binding to its catalytic residue (reviewed in Chen et al. 1999; Farahat et al. 2011). Therefore, we investigated the possibility that dioxin affects the enzymatic activity of AChE in cultured neurons and further explored the possibility that this occurs by a transcriptional mechanism.

Dioxin is thought to exert its biological and toxicological effects primarily by binding to the aryl hydrocarbon receptor (AhR, dioxin receptor) followed by nuclear translocation and binding to response elements (DREs) in gene promoters (reviewed in Beischlag et al. 2008). Putative DREs were present in the promoter of the human *ACHE* gene, but not in the mouse or rat *ACHE* genes (Sun et al. 2004), suggesting the possibility of species-specific effects of dioxins on AChE. Therefore, the role of the AhR-dependent pathway in dioxin-induced alterations of AChE and the species specificity of the effects were also studied.

## Materials and Methods

**Cell culture.** SK-N-SH, a cell line derived from human neuroblastoma cells, was purchased from the cell resource center of Chinese Academy of Medical Sciences. These cells express both AChE and muscarinic acetylcholine receptor (Ezoulin et al. 2008; Pizzi et al. 2002; Popova and Rasenick 2004). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a water-saturated 5% CO<sub>2</sub> incubator. PC12, a cell line derived from a pheochromocytoma of the rat

adrenal medulla, was maintained in DMEM supplemented with 6% FBS and 6% heat inactivated horse serum, and incubated at 37 °C in a water-saturated 5% CO<sub>2</sub> incubator. All reagents for cell culture were from Invitrogen (Carlsbad, CA).

**Chemical treatment.** The cells were seeded in 6-well-plates at 500,000 cells per well 24 hours before exposure to dioxin or other drug treatment for AChE activity determination. The most potent congener of dioxins, TCDD, was purchased from Wellington Laboratories Inc. (Ontario, Canada) and employed at low concentrations of  $10^{-11} \sim 10^{-9}$  M. CH223191, an inhibitor of the AhR-dependent pathway (Zhao et al. 2009), was obtained from Sigma (St. Louis, MO) and employed at  $10^{-5}$  M. A pre-treatment with CH223191 3 hours before incubation with TCDD was employed to study the role of AhR. The solvent dimethyl sulfoxide (DMSO) was present at 0.1% for all treatments. Treated cultures were compared with cultures exposed to 0.1% DMSO alone or other indicated groups. In the *in vitro* assay, SK-N-SH cell lysate was incubated with TCDD ( $10^{-11}$  to  $10^{-9}$  M), with BW284c51 (Sigma) at  $2 \times 10^{-5}$  M or with solvent alone at 0.1% (Control). After one-hour incubation at room temperature, enzymatic activity of AChE was determined by the Ellman assay (Ellman et al. 1961). BW284c51, a specific inhibitor of AChE, serves as an assay control.

**Reporter gene constructs and transfections.** pAChE-Luc and pAChEm-Luc consist of the human *ACHE* and mouse promoter sequences upstream of a firefly luciferase gene in pGL4.10 and pGL3-Basic vectors (Promega, Madison, WI), respectively. The truncated construct, pAChE-T-Luc, derived from pAChE-Luc, was constructed using sense primer 5'- TTA GAT CTC CTC AGG TGA GTC TC -3' and antisense primer 5'-TTA AGC TTG GCT GCA GGG

CAG -3'. BglIII and HindIII restriction sites were added at the 5' ends of sense and antisense primers, respectively, as indicated (underlined). The mutated construct, pAChE-M-Luc, derived from pAChE-Luc, was constructed by site-directed mutagenesis, accomplished using mutagenic primers and flanking primers. The mutagenic primers include sense primer, 5' -GTC CGT CTG CGA ATT CTC TGT CTC C -3', and anti-sense primer, 5' -GGA GAC AGA GAA TTC GCA GAC GGA C -3', where the original sequence of the putative DRE (5' -GCG TG- 3') was replaced by an EcoRI restriction site (5' -GAA TTC- 3') as indicated (underlined). The flanking primers included sense primer, 5' -TTA GAT CTA GAT CTC GAG CTC GAG GAT CCC- 3', and anti-sense primer, 5' -TTA AGC TTC GCC TGC CCT GCA GCC AAG CTT-3', where BglIII and HindIII restriction sites were added at the 5' ends of primers, respectively, as indicated (underlined). PCR was performed using Pfx polymerase (Invitrogen) and the product was sub-cloned into the same vector as the full length pAChE (pGL4.10) via BglIII and HindIII restriction sites to produce truncated construct pAChE-T-Luc and mutated construct pAChE-M-Luc. Cultured cells were seeded in 24-well-plates at 50,000 cells per well 24 hours before being transfected transiently with purified plasmids (0.5 µg per well) and PolyJet<sup>TM</sup> reagent (SigmaGen Laboratories, Rockville, MD) according to the manufacturer's instructions. The transfection efficiency was ~15%.

***Determination of AChE enzymatic activity.*** AChE enzymatic activity was determined according to the method of Ellman (Ellman et al. 1961), modified by the addition of 0.1 mM tetra-isopropylpyrophosphoramidate (iso-OMPA), an inhibitor of butyrylcholinesterase (BChE). Cells were collected and total protein extraction was performed at 20 °C for 15 min in 200 µL of

low salt lysis buffer (80 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4) supplemented with 0.5% Triton X-100 and 2.5 mM benzamidine, a protease inhibitor. About 30  $\mu\text{L}$  cell lysates were incubated with 0.1 mM iso-OMPA and 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 30 min at 20 °C to inhibit the BChE activity and allow saturation of unspecific reaction with DTNB, followed by addition of 0.625 mM acetylthiocholine iodide to start the AChE-specific reaction. Absorbance at 410 nm was recorded with a multi-functional microplate spectrometer (TECAN Infinite F200 pro, Switzerland). Optical density (OD) was recorded at 5 min intervals over a period of 30 min, at 20 °C. In this period of time, OD derived from the cell lysate was observed to increase linearly with time. The velocity of the reaction was calculated from the slope of the line obtained. Arbitrary units of enzymatic activity were expressed as velocity (mOD per minute) per  $\mu\text{g}$  of protein. All reagents were obtained from Sigma. Protein concentrations were measured by the Bradford method with a kit from Bio-Rad Laboratories (Hercules, CA).

***Luciferase assay*** Cells were transfected with promoter-reporter constructs together with cDNA encoding the  $\beta$ -galactosidase gene at 10:1 weight ratio. Twenty-four hours later, cells were treated with chemicals as described in the preceding Chemical treatment section. For luciferase measurement, sample wells were washed twice with phosphate-buffered saline, followed by addition of cell lysis buffer (Promega) and shaking of the plates for 10 min at room temperature to allow cell lysis. Insoluble material was removed by centrifugation, and the resulting lysates were transferred to white 96-well microplates for measurement of luciferase activity, using a TECAN Infinite F200 Pro luminometer with automatic injection of Promega

stabilized luciferase reagent. Luciferase activity in each well was normalized to total protein amount and to transfection efficiency as determined by  $\beta$ -galactosidase activity.

***Real-time quantitative PCR.*** Total RNA (5  $\mu$ g) from SK-N-SH cultures was isolated using TRIzol reagent (Invitrogen), and cDNA was prepared using 5  $\mu$ g of RNA and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR of AChE T subunit (AChE<sub>T</sub>), AChE R subunit (AChE<sub>R</sub>), PRiMA (proline-rich membrane anchor) and 18S rRNA transcripts was performed on equal amounts of cDNA, using SYBR Green Master mix and Rox reference dye, according to the manufacturer's instructions (Applied Bioscience, Foster City, CA). The primers were: 5'- GGG GTT CCC CAG GTA AGT GAC CT C -3' and 5'-T TG AGC AGC GAT CCT GCT TGC TGT AG -3' for human AChE<sub>T</sub> transcript (NM\_000665), 5'-CTG GGG TGC GGA TCG GTG TAC CCC-3' and 5'-TCA CAG GTC TGA GCA GCG TTC CTG-3' for rat AChE<sub>T</sub> (Boudreau-Larivière et al. 2000), 5'- CCC CTG GAC CCC TCT CGA AAC -3' and 5'- TGG GGA GGA AGC GGT TCC AGA AG -3' for human AChE<sub>R</sub> transcript (AY750146; Birikh et al. 2003), 5'- TCT GAC TGT GCT TGT CAT CAT TTG CTA C -3' and 5'- AGG GCC TGC AGA CTC ACA CCA C-3' for human PRiMA transcript (NM\_178013) and 5'- GAC TGT TAT GGT CAA GGT GAA-3' and 5'-GAT AGT CAA GTT CGA CCG TC-3' for human 18S rRNA (NR\_003286; Guo et al. 2011). The SYBR green signal was detected by Mx3005p multiplex quantitative PCR machine (Stratagene, La Jolla, CA). The relative transcript expression levels were quantified using the  $\Delta\Delta$ Ct method (Winer et al. 1999). The specificity of amplification was confirmed by melting curves and by gel electrophoresis.

**MTT assay.** In cell viability tests, cultured SK-N-SH cells in 96-well-plates were treated with TCDD for 48 hours followed by addition of 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) in PBS at final concentration of 0.5 mg/ml for 2 hours. The medium was aspirated, and the cultures were re-suspended in 150  $\mu$ L DMSO to determine cell viability by absorbance at 570 nm.

**Other assays.** For assay of  $\beta$ -galactosidase enzymatic activity, 20  $\mu$ L of lysates were mixed with 80  $\mu$ L of sodium phosphate buffer (pH 7.5) containing 0.8 mg/ml o-nitrophenyl- $\beta$ -D-galactopyranoside. After incubation at 37 °C for 1 hour, absorbance was measured at 410 nm. In this period of time, OD derived from the cell lysate had been verified to vary linearly with time.

**Statistics.** Statistical tests were made by the Origin Pro program (version 8, OriginLab): One-way ANOVA was employed in most data analyses to assess differences from controls or differences from other treatments, while two-way ANOVA was employed in promoter truncation and mutation study. Bonferroni test was used for means comparisons between two treatment groups. Differences were classified as significant \* (from control) or # (from other treatment) for  $p < 0.05$ .

## Results

**Dioxin decreases the enzymatic activity of neuronal AChE.** To investigate effects of dioxin on neuronal AChE activity, quiescent human SK-N-SH neuroblastoma cells were exposed to TCDD ( $10^{-11} \sim 10^{-9}$  M) for 48 hours. No obvious cell death was observed as assessed by MTT

assays (Figure 1A). Effects of treatment, time, and TCDD concentration on the enzymatic activity of AChE were determined. Compared to DMSO treatment ( $2.8 \pm 0.18$  mOD/mim/ $\mu$ g), the enzymatic activity of AChE was reduced by approximately 15% after 24-hour exposure to  $10^{-10}$  M and  $10^{-9}$  M TCDD (Figure 1B). The lowest concentration of TCDD ( $10^{-11}$  M) had no effect on AChE activity (Figure 1B). Because the  $10^{-10}$  M and  $10^{-9}$  M groups did not differ, we used  $10^{-9}$  M of TCDD for the time course experiments. Significant decreases in AChE activity were observed after 12, 24 and 48 hours of exposure to  $10^{-9}$  M TCDD. There were no differences between effects at the various time points. In time course experiments, the AChE activity in all the control groups varied with the time. The mean value of controls in 6~24-hour groups varied little ( $2.8 \sim 3.1$  mOD/mim/ $\mu$ g) and that of 48-hour group was relatively high ( $5.7 \pm 0.08$  mOD/mim/ $\mu$ g). The relatively high AChE activity in 48-hour group might be due to the higher cell density of the cultures at harvest. Moreover, other dioxins such as 2,3,7,8-tetrachlorodibenzofuran (TCDF;  $\sim 74\%$  of control) and 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF;  $\sim 68\%$  of control) also decreased AChE activity in SK-N-SH cells (see Supplemental Material, Table S1).

***Dioxin does not directly inhibit AChE enzyme.*** So far we have found dioxin decreased AChE activity, so we next determined whether dioxin could directly inhibit the enzymatic activity of AChE using an *in vitro* assay. TCDD was mixed with cell lysate of SK-N-SH cultures for 1 hour at 20 °C. None of the TCDD concentrations employed ( $10^{-11} \sim 10^{-9}$  M) inhibited the activity, while addition of BW284c51 ( $2 \times 10^{-5}$  M), a specific AChE inhibitor, to the cell lysate inhibited nearly 90% of the enzymatic activity (Figure 2A). These results suggested that dioxin is

unlikely to act by direct inhibition of AChE and that the suppressive effects of dioxin on AChE activity may occur via a transcriptional regulation.

***Dioxin suppresses AChE activity via AhR.*** When AhR is activated by dioxins, it will translocate into the nucleus and form a heterodimer with its partner ARNT (aryl hydrocarbon receptor nuclear translocator). The heterodimer binds to DRE in the promoter region upstream of target genes to regulate the transcription. The role of AhR in the dioxin-induced decrease of AChE activity was first investigated using CH223191, a ligand-selective antagonist of the AhR, which can preferentially inhibit effects of certain classes of AhR agonists, including TCDD (Zhao et al. 2010). Compared to DMSO treatment ( $3.8 \pm 0.18$  mOD/mim/ $\mu$ g), TCDD ( $10^{-9}$  M) treatment significantly decreased the activity (Figure 2B), consistent with the result in Figure 1B. In contrast, pretreatment with CH223191 blocked the decrease by TCDD, indicating that AhR was involved in the dioxin-induced effect.

***Dioxin causes transcriptional down-regulation of AChE.*** A human *ACHE* promoter-driven luciferase reporter construct (pAChE-Luc) with  $\sim 2.2$  kb of the regulatory region upstream of the human *ACHE* gene was employed to evaluate TCDD effects on the promoter activity of human AChE. This construct has been well characterized and extensively used to study the regulation of *ACHE* gene (Getman et al. 1995; Siow et al. 2002). Quiescent SK-N-SH cells were transiently transfected with pAChE-Luc one day before the application of TCDD ( $10^{-11} \sim 10^{-9}$  M). The promoter activity was determined by luciferase assay after 24-hour of TCDD-treatment. Consistent with AChE activity, a significant decrease ( $\sim 30\%$ ) in human *ACHE* promoter activity was found upon TCDD ( $10^{-10} \sim 10^{-9}$  M) exposure as compared to DMSO treatment (Figure 3A).

Similar to the effects on AChE activity, pre-treatment with CH223191 ( $10^{-6}$  M) significantly reversed the suppressive effect of TCDD ( $10^{-9}$  M) on the promoter activity of human *ACHE*, consistent with our assumption that an AhR-dependent pathway directs the dioxin-induced transcriptional suppression of AChE (Figure 3B).

The transcriptional regulation of AChE by dioxin was further confirmed by real time PCR analyses to determine expression levels of AChE<sub>T</sub> mRNA, the major AChE transcript in neurons, AChE<sub>R</sub> mRNA, the minor AChE transcript in the brain and PRiMA mRNA, a structural subunit of the active form of neuronal AChE (reviewed in Massoulié, 2002). Results showed a ~25% decrease in AChE<sub>T</sub> mRNA level in response to TCDD exposure at  $10^{-9}$  M, without any significant changes in AChE<sub>R</sub> and PRiMA mRNA levels (Figure 3C). Similar to the change in AChE mRNA, the protein level of AChE was obviously reduced after TCDD exposure (See Supplemental Material, Figure S1). Therefore, we concluded that exposure to dioxin leads to a decrease in the mRNA expression of AChE catalytic subunit resulting in decreased expression of the active form of AChE.

***Putative DRE(s) on human AChE promoter.*** The presence of DREs in the regulatory region upstream of dioxin-responsive genes is a key component of the AhR-dependent signaling pathway in response to dioxin. Four putative consensus core sequences of DRE (5'-TNGCGTG-3' or 5'-CACGCNA-3') (Nukaya et al. 2009) were found within the ~2.2 kb region upstream the human *ACHE* gene (Gene Bank: NM\_000665). All of the putative DREs are exact matches or reverse complements of 5'-GCGTG-3' (See Supplemental Material, Figure S2). Considering the 5' DRE is the only one located upstream of the transcription start sites among all four putative

DREs (Getman et al. 1995; Figure 4, upper panel), we concentrated our investigation on the role of this putative DRE. In cultures transfected with truncated human AChE promoter missing the 5' DRE site (pAChE-T) or with a promoter containing a specific mutation of the DRE site (pAChE-M), we found TCDD no longer suppressed the promoter activities (Figure 4, lower panel). Furthermore the effect of TCDD on pAChE-M was significantly different from that of wild type at concentrations of  $10^{-10}$  M and  $10^{-9}$  M. These results suggest that the 5' DRE may play a critical role in mediating the suppression.

***Differential responses of rodent AChE to dioxin.*** Since rodent AChE genes lack obvious DREs (Sun et al. 2004; See Supplemental Material, Figure S2), we tested effects of dioxin on AChE in PC12 cells, a widely-used rat neuronal cell line in toxicology studies. After a 24-hour exposure to TCDD ( $10^{-10}$ ,  $10^{-9}$  and  $10^{-8}$  M), none of the treatment groups showed suppression of the AChE activity (Figure 5A). Similarly, TCDD exposure had no effect on the promoter activity of mouse *ACHE* by using a construct (pAChEm-Luc) consisting of a ~2.1 kb regulatory region upstream the mouse *ACHE* gene driving a luciferase reporter gene expression (Figure 5B; Jiang et al. 2003). Absence of consensus DRE sequences in the 2.1 kb regulatory region upstream of the mouse *ACHE* gene may help to explain the different responses of rodent and human AChE genes (Sun et al. 2004). PC12 cells employed in the study exhibited normal responses to other drug treatments, such as forskolin, which activates cAMP-dependent pathway and nerve growth factor. Neurite outgrowth and an increment in AChE activity could be observed in the treated cells, respectively (See Supplemental Material, Figure S3). These data further support that the unresponsiveness to TCDD may be due to the absence of DRE on the promoter of rodent *ACHE*.

## Discussion

Emerging evidence has shown effects of dioxin in the central cholinergic system. Ahmed (2011) reported that daily administration of TCDD (0.2 or 0.4 µg/kg body weight) to pregnant rats from gestation day (GD) 1 to lactation day (LD) interfered with the development of AChE expression in cerebella of the offsprings, although there was no obvious sign of developmental toxicity. Ahmed (2011) suggested that the effects of TCDD on brain AChE were related to alterations in thyroid development. This *in vivo* study suggested TCDD is able to affect AChE in the brain. The present *in vitro* study showed that TCDD suppressed AChE activity in cultured human neuronal cells. However, AChE may not be the only target of dioxin in cholinergic system, since putative DRE sites are also present in the promoter region of acetylcholine receptor as well (Sun et al. 2004). Thus, the present finding on AChE is a new start to explore more abnormalities in cholinergic function directly caused by dioxin.

Besides the classical function of AChE in the cholinergic neurotransmission, more and more evidence has shown that AChE may have other functions, such as in synapse transmission (reviewed in Zimmerman and Soreq 2006), neurite outgrowth (reviewed in Paraoanu and Layer 2008), apoptosis (reviewed in Jiang and Zhang 2008), bone formation (Vogel-Hopker et al. 2012). Thus the interference of dioxin with AChE might bring new insight into the biological or toxicological effects mediated by AhR not only in nervous system but elsewhere in the human body.

Monitoring of AChE inhibition has been used as an indicator of OP exposure. Emerging evidence suggests that other types of xenobiotics, such as heavy metals and nanoparticles, can also affect AChE activity (Richetti et al. 2011; Wang et al. 2009). Here we provide evidence of neuronal AChE regulation by another type of xenobiotic, dioxins. The present findings showed that AhR mediated the transcriptional regulation of AChE. Considering of the ability of AhR to bind diverse structures of chemicals (Zhao et al. 2010), the spectrum of xenobiotics affecting AChE transcription could extend beyond dioxin-like chemicals. These results suggest that in the future, in addition to assay for AChE activity, it may be possible to monitor exposures by demonstrating down-regulation of AChE transcripts.

In our experiments, the alterations in neuronal AChE expression were produced by TCDD exposure at low concentrations, close to environmental levels. In several accidental exposures to dioxins, such as in Vietnam, Seveso Italy and Taiwan, exposed individuals had median serum levels of ~1000, 450 and 180 pg/g fat, respectively (Guo et al. 2004; Needham et al. 1997; Tai et al. 2011). By calculation with the estimated average fat content in the serum at 6.9 g/L (Phillips et al. 1989), the average serum concentration of dioxin (TCDD) in these individuals would be within  $10^{-10} \sim 10^{-11}$  M. Although concentrations of dioxins in brain tissue of exposed individuals are unknown, we based our experimental concentrations on these serum concentrations and on concentrations used in other studies (Sánchez-Martín et al. 2010; Jin et al. 2004). In the present study, using relatively low concentrations of TCDD, the cultured neuronal cells exhibited no significant change in viability, which made it feasible to study the functional alterations induced by dioxin.

Based on the present findings and the literature, we assume the molecular events involved in the effect of dioxin are as follows: when dioxin enters the neuronal cells, it will bind to AhR in the cytosol, which results in the transformation and translocation of the receptor. The active AhR then goes into the nucleus and binds with ARNT to form a heterodimer. The heterodimer will then bind to the putative DRE site(s) on the promoter region of the *ACHE* gene, suppressing the expression of AChE<sub>T</sub> transcripts (reviewed in Beischlag et al. 2008). This transcriptional suppression of the major neuronal AChE transcript leads to a decrease in the production of the AChE catalytic subunits and finally causes the decrease in enzymatic activity. However, further investigations on the role of the putative DRE(s) and how the transcriptional suppression occurs are needed. Apart from this transcriptional mechanism, we did not find any evidence showing that dioxin could inhibit the AChE activity by direct interaction with the catalytic subunit.

Toxic effects of environmental chemicals can be species specific. For example, the binding affinity of dioxin to AhR is higher in mice than in humans (reviewed in Denison et al. 2011), and endocrine-disrupting chemicals, such as bisphenol A and its analogues, are potent agonists for human pregnane X receptor (hPXR) but do not affect mouse PXR activity (Sui et al. 2012). Our findings here show that dioxin has a suppressive effect on AChE expression in human, but not in rat, neuronal cells (Figure 5). The species specificity found in the present study is unlikely to be due to differences in affinities of dioxin-AhR binding, but rather resulted from the presence and absence of DREs in the regulatory regions of human and rodent *ACHE* genes, respectively. This finding highlights the advantage of *in vitro* toxicity testing using cell lines derived from human over animal testing when trying to assess effects of human exposure to

xenobiotics, as proposed by Tox21 (Bhattacharya et al. 2011; Hartung 2009; reviewed in Krewski et al. 2010).

## **Conclusion**

We found a novel mechanism whereby dioxin may produce its biological or toxicological effects by decreasing neuronal AChE activity through a transcriptional down-regulation mechanism via the AhR-dependent pathway. To our knowledge, this is the first study to report direct interference by dioxin with the cholinergic neurotransmission system.

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## Figure legends

**Figure 1.** Suppressive effects of TCDD to the enzymatic activity of AChE in neurons. Cultured SK-N-SH cells were incubated with TCDD ( $10^{-11}$  to  $10^{-9}$  M) or with solvent alone at 0.1% (Control) for 6 to 48 hours. Cell viability and the enzymatic activity of AChE were determined as described in Materials and Methods. (A) Forty eight hours after the exposures, cell viability was assessed by MTT assay. Dose (B) and time (C) effects of TCDD are shown, in which TCDD suppresses AChE activity in cultured neuronal cells. Values are calculated as % of solvent alone and expressed as mean $\pm$ SEM,  $n=4$ , each independent sample was tested in triplicate. Bonferroni test was used for means comparisons between two treatment groups;  $*p<0.05$ , difference from solvent-treated cells (Control) by one-way ANOVA with Bonferroni test;  $\#p<0.05$ , difference from TCDD-treated cells at concentration of  $10^{-11}$  M in (B) by one-way ANOVA with Bonferroni test.

**Figure 2.** TCDD suppresses AChE activity via AhR-dependent pathway, but not via direct inhibition to the enzyme. (A) Cell lysate of SK-N-SH was incubated with TCDD ( $10^{-11}$  to  $10^{-9}$  M), with BW284c51 at  $2 \times 10^{-5}$  M or with solvent alone at 0.1% (Control). After one-hour incubation, enzymatic activity of AChE was determined by the Ellman assay. BW284c51, a specific inhibitor of AChE, serves as a positive control. (B) Cultured SK-N-SH cells were incubated with the AhR inhibitor, CH223191 at  $10^{-6}$  M, or with solvent alone at 0.1% for 3 hours prior to addition of TCDD at  $10^{-9}$  M or solvent alone at 0.01%. After 24 hour incubation, the enzymatic activity of AChE was determined. Values are calculated as % of solvent alone and

expressed as mean $\pm$ SEM,  $n=4$ , each independent sample was tested in triplicate. Bonferroni test was used for means comparisons between two treatment groups;  $*p<0.05$ , difference from solvent-treated cells (Control) by one-way ANOVA with Bonferroni test;  $\#p<0.05$ , difference from TCDD-treated alone in (B) by one-way ANOVA with Bonferroni test.

**Figure 3.** TCDD-induced suppression of AChE occurs through a transcriptional down-regulation mediated by AhR-dependent pathway. The human *ACHE* promoter-reporter construct (pAChE-Luc) was transiently transfected into cultured SK-N-SH cells one day before drug treatment. After one-day-treatment, luciferase assays were performed to determine the promoter activity of human AChE (A, B). (A) The transfected cells were incubated with TCDD ( $10^{-11} \sim 10^{-9}$  M) or with solvent alone at 0.1% (Control). (B) The transfected cells were subjected to pre-treatment of CH223191 plus treatment of TCDD ( $10^{-9}$  M) as in Figure 2 (B). (C) Total RNA was extracted from TCDD ( $10^{-9}$  M) or solvent treated culture and real time PCR analyses were performed to determine the expression level of AChE transcripts (AChE<sub>T</sub> and AChE<sub>R</sub> variant) and PRiMA transcripts. Values are calculated as % of solvent alone and expressed as mean $\pm$ SEM,  $n=3$ , each independent sample was tested in triplicate. Bonferroni test was used for means comparisons between two treatment groups;  $*p<0.05$ , difference from solvent-treated cells (Control) by one-way ANOVA with Bonferroni test;  $\#p<0.05$ , difference from TCDD-treated group by one-way ANOVA with Bonferroni test.

**Figure 4.** Putative DRE consensus sequences in human *ACHE* promoter. Four putative DRE consensus sequences in the promoter are shown in the upper panel. A fragment consisting of the

promoter sequences from -1 to -1568 and a fragment with mutation on the 5' putative DRE were obtained by PCR as mentioned in Materials and Methods. The PCR products were subcloned into pGL4.10 vector to produce a truncated promoter-reporter construct pAChE-T-Luc and a mutated promoter-reporter construct pAChE-M-Luc (Upper panel). These two constructs and the full length pAChE-Luc construct were transiently transfected into cultured SK-N-SH cells separately one day before being incubated with TCDD ( $10^{-10} \sim 10^{-9}$  M) or with solvent alone at 0.1% (Control) for 24 hours. Luciferase assays were then performed to determine the promoter activity. Values are calculated as % of solvent alone and expressed as mean $\pm$ SEM,  $n=3$ , each independent sample was tested in triplicate. Bonferroni test was used for means comparisons between two treatment groups; \* $p<0.05$ , difference from solvent-treated cells (Control) by one-way ANOVA with Bonferroni test; # $p<0.05$ , difference from pAChE-Luc transfected cells by two-way ANOVA with Bonferroni test.

**Figure 5.** Suppressive effect of TCDD on AChE is not present in rodents. (A) Cultured PC12 cells were incubated with TCDD ( $10^{-10} \sim 10^{-8}$  M) or with solvent alone at 0.1% (Control) for 24 hours for determination of the AChE enzymatic activity as described in Figure 1. (B) The mouse *ACHE* promoter-reporter construct (pAChEm-Luc) was transiently transfected into cultured PC12 cells and exposed to TCDD ( $10^{-10} \sim 10^{-8}$  M) for the determination of promoter activity as described in Figure 3 (B). Values are calculated as % of solvent alone and expressed as mean $\pm$ SEM,  $n=3$ , each independent sample was tested in triplicate.

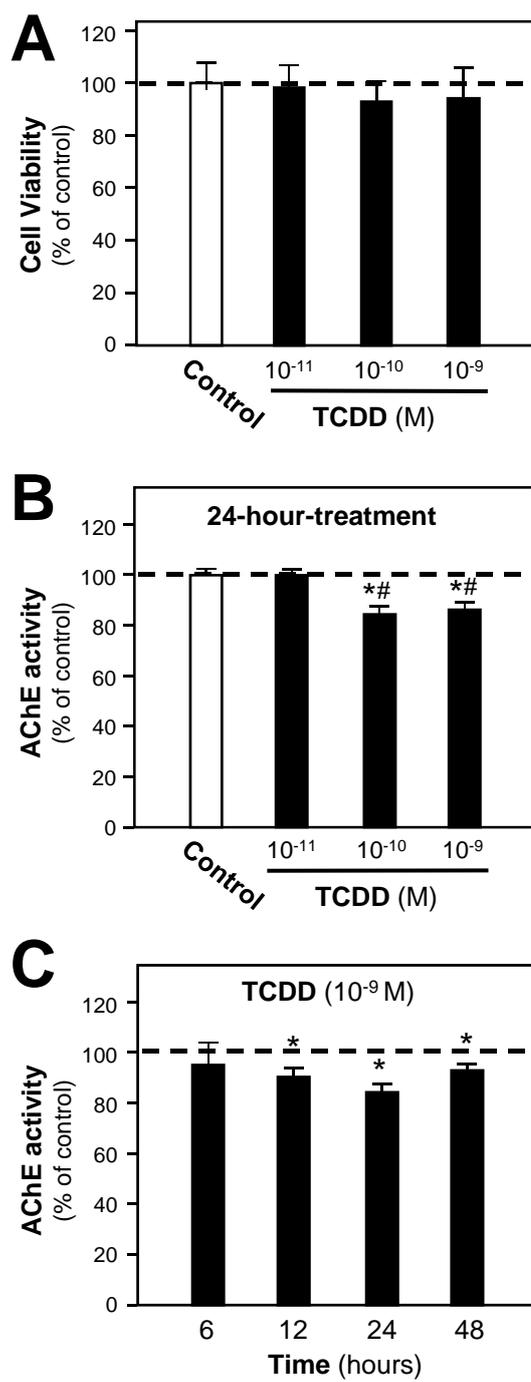


Figure 1

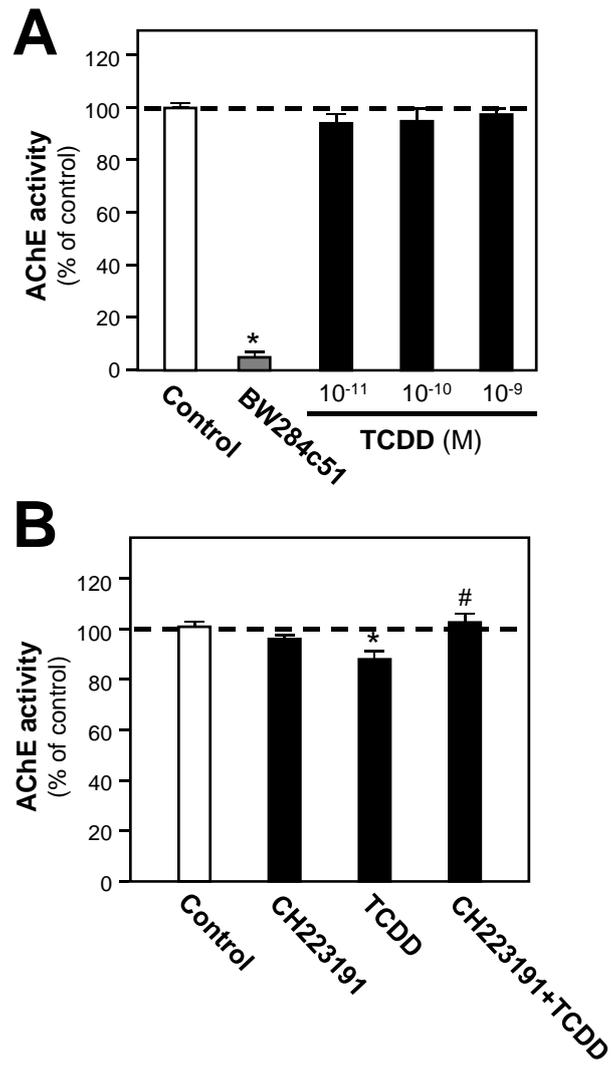


Figure 2

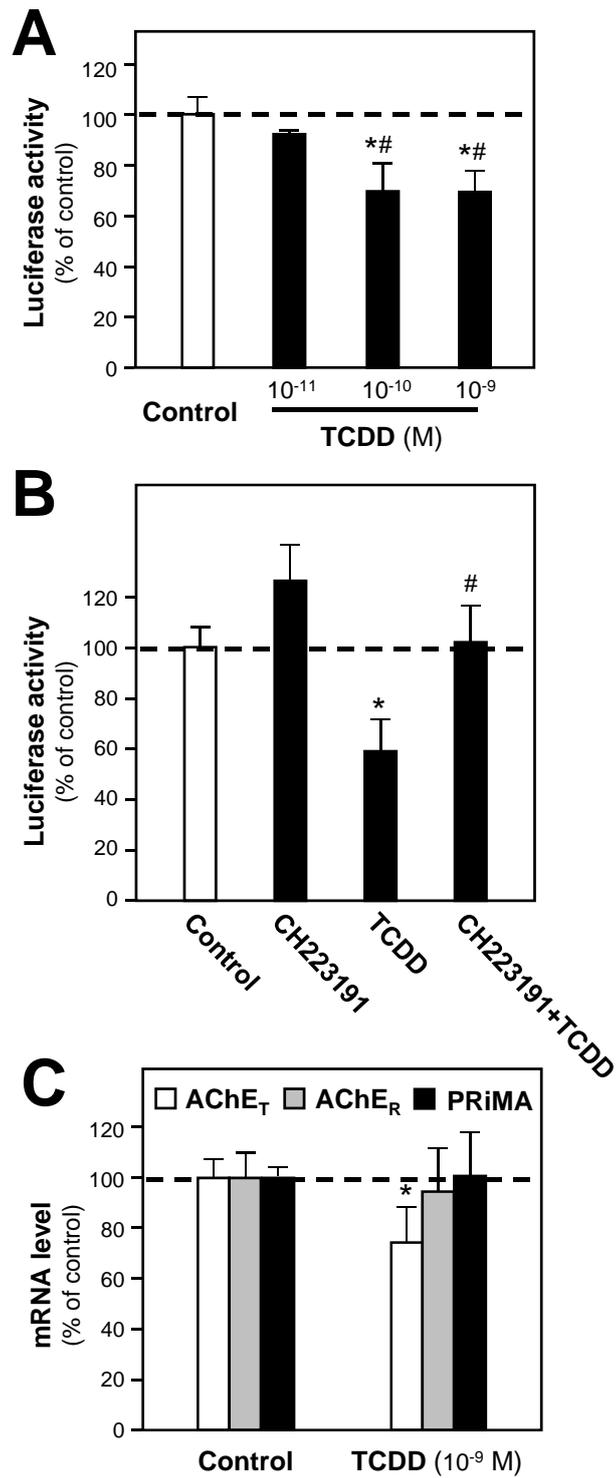


Figure 3

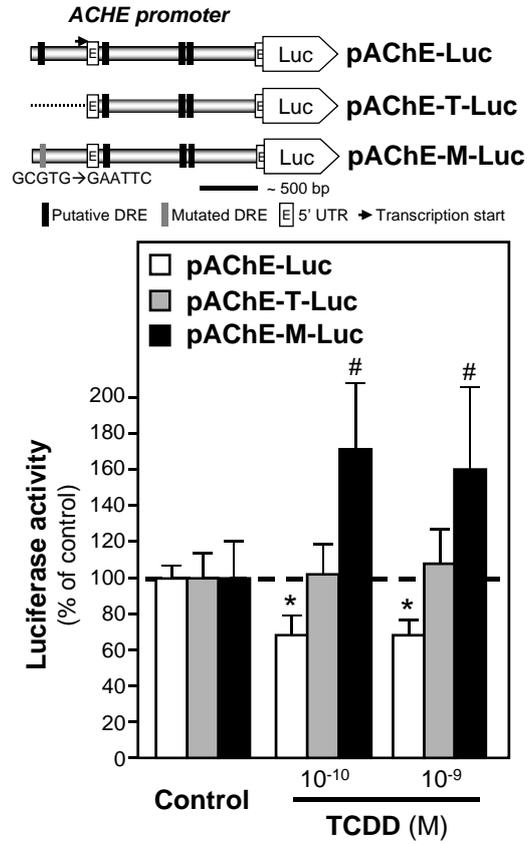


Figure 4

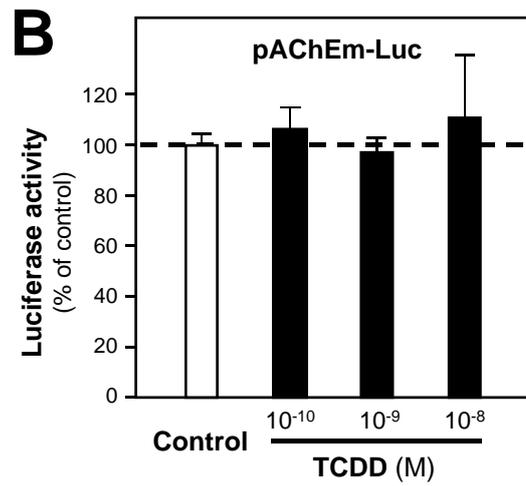
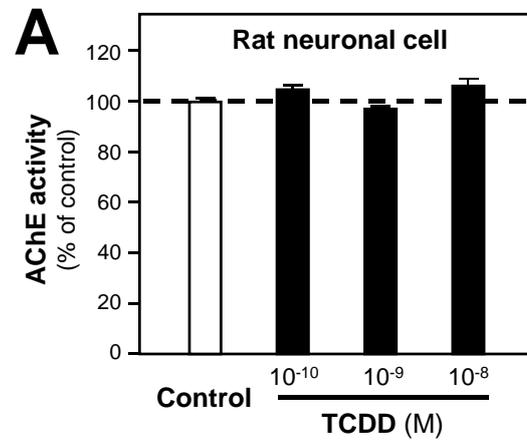


Figure 5