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Article

# Astrocytes Respond to Environment-Relevant Doses TCDD in a Specific Manner Which Is Different from the Endogenous AhR Ligand (FICZ)

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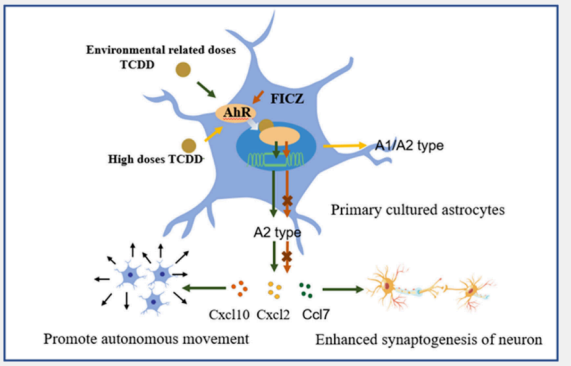
**ABSTRACT:** Astrocytes play an important role in the nervous system's response to external stimulation. Environmental pollutants could activate astrocytes into A1 (toxic) or A2 (protective) types and induce different effects. Meanwhile, the aryl hydrocarbon receptor (AhR) is an environmental molecule sensor in the body and has various ligands. But the difference between exogenous and endogenous AhR ligands on the astrocytic activation is unclear; in this study, we employed rat primary cultured cortical astrocytes to reveal the effects and mechanisms of AhR ligands on astrocytic activation. We found that, after treatment with exogenous AhR ligand (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) ranging from 0.01 to 0.1 nmol/L, astrocytes mainly exhibited A2 type activation. The specific manifestation includes the increase in the expression of A2 marker genes, the enhancement of cellular autonomous movement, the expression and secretion of chemokines, such as Cxcl10, Cxcl2, and Ccl7. And TCDD-induced A2 type astrocytes show a positive impact on neuronal synaptic formation. Although both TCDD and endogenous AhR ligand (6-formylindolo[3,2-*b*]carbazole, FICZ) could activate AhR pathway in astrocytes, FICZ (50 nmol/L) neither induces activation of A2 type astrocytes nor upregulation of chemokines. Therefore, our findings suggest that AhR is crucial for astrocytes to recognize environmental pollutants and protect the nervous system.




**KEYWORDS:** *2,3,7,8-tetrachlorodibenzo-p-dioxin, 6-formylindolo[3,2-b] carbazole, astrocytic activation, aryl hydrocarbon receptor, the nervous system*

## INTRODUCTION

Astrocytes are the largest cell population in the brain; they interact with other cells closely, like neurons and microglia. The functions of the astrocyte include scavenging compounds, repairing damaged neurons, and participating in the innate immune responses or neuroinflammatory responses.[1,2] They also interact with local neural circuits and play roles in information processing and generating physiological and behavioral responses.[3] It is believed that astrocytes are crucial in responding to environmental pollutants, such as dioxins and dioxin-like compounds (DLCs).[4,5] Emerging data suggests that the most toxic dioxin congener 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces the activation of astrocytes, but the consequences of the activation and the action mechanisms have yet to be elucidated.[6]

The different activation phenotypes of reactive astrocytes have been characterized and defined as A1 and A2 type, respectively, depending on the harm or protective effect on neurons after activation. A1 reactive astrocytes contained the marker genes and neurotoxic mediators (such as TNF-$\alpha$, IL-6, IL-8, Ccl2, etc.), which would damage the synapses in the related neurons.[7,8] Whereas in A2 astrocytes, neurotrophic factor and immune regulatory genes (Cxcl10, IFN-$\beta$) are upregulated to repair synapses and protect neurons.[9] Cytokines and chemokines produced by the reactive astrocytes are crucial to immunomodulation and neuron protection after brain injury, the alterations in the synaptic transmission and the ultimate functional changes in neurons.[10,11] Meanwhile, these chemokines secreted by reactive astrocytes can recruit astrocytes themselves and other cells (including neuron, microglia, and white blood cells) by enhancing cell motility.[12] Thus, to evaluate the activation types of astrocytes, it is necessary to elucidate the response status of astrocytes after exposure to dioxins and their subsequent effects on interacting neurons.

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In the previous study, Chen et al. found 1 nmol/L of TCDD could promote the migratory ability of astrocytes and upregulate the pro-migratory gene expression via aryl hydrocarbon receptor (AhR) pathway.[13] AhR is a ligand dependent transcription factor that mediates the toxic and physiological effects of various environmental pollutants (such as dioxins and DLCs) and endogenous compounds. After binding with ligands, AhR is activated into the nucleus to trigger transcriptional regulation of downstream genes, including cytochrome P450 family genes and AhR repressor (AHRR) gene.[14,15] Among these ligands, TCDD is the most potent exogenous agonist and is usually used as a representative compound to study the toxicological effects and action mechanisms. In primary cultured astrocytes, TCDD induced the expression of AhR pathway downstream classic response genes CYP1A1 and CYP1B1,[16] indicating that the AhR pathway is a critical molecular mechanism for the action of dioxins on astrocytes. In this study, we aim to investigate the effects of TCDD on astrocytes at environment-relevant concentrations (0.003−0.3 nmol/L),[17] including the reactive status, synaptic molecules dysfunction, chemokine production, and autonomous motility. At the same time, we also involved the effect of the endogenous AhR ligand 6-formylindole [3,2-*b*] carbazole (FICZ) on the activation status of astrocytes to reveal the ligand specificity of AhR. This study indicates that external TCDD stimulation is crucial for the response types of astrocytes and their functions in the nervous system. The astrocytic reactivation data obtained in the study may provide new insights into the self-protection mechanisms by which the nervous system responds to exogenous pollutants through astrocytes.

## MATERIALS AND METHODS

### Reagents and Antibodies

TCDD (DD48D0512, purity ≥98%) dissolved in dimethyl sulfoxide (DMSO) was purchased from Wellington (Ontario, Canada). FICZ (SML1489, purity ≥95%) was purchased from sigma (St. Louis, MO). Reagents including DMSO (D8418) and CH223191 (C8124) were obtained from Sigma (St. Louis, MO). FICZ and DEMO were dissolved by DMSO. P450-Glo CYP1A1 Assay System (V8752) was purchased from Promega (Madison, WI). Cxcl2 ELISA Kit (ab155463) and Ccl7 ELISA Kit (ab205571) were purchased from Abcam (Cambridge, MA). Cxcl10 ELISA Kit (KA2203) was purchased from Abnova (Taoyuan, Taiwan). Anti-Synaptotagmin-1 (D33B7) and anti-PSD95 (D27E11) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

### Cell Culture

The isolation and culture method for cortical astrocytes and neurons is described in detail in Supporting Information. The average purity of primary cultured cortical astrocytes for subsequent experiments was higher than 95%. Three hours before each chemical treatment, the culture medium was replaced with low serum medium (MEM with 1% horse serum, 100 units/mL penicillin, and 100 μg/mL streptomycin). All animal experiment were approved by the Animal Ethics Committee of the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (permission number: AEWC-RCEES-2022006).

### Transcriptomic Analysis and Quantitative Real-Time PCR (qPCR)

The total mRNA of astrocytes and neurons were extracted by the Thermo Scientific GeneJET RNA Purification Kit (K0732, Thermo Scientific, Baltics, Turkey). For microarray, astrocytes mRNA was analyzed on expression console at Gene company limited. DEGseq was used to analyze differentially expressed genes between DMSO and TCDD groups.[18] The clustering genes ($\log_2$Ratio(T/C) ≥ 1.5) of DMSO group and TCDD group were subjected to GO enrichment analysis using DAVID online software. For qPCR, 1 μg total mRNA was reverse transcript into cDNA with RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific). Ten fold diluted cDNA was subjected to PCR cycles by using the GoTaq qPCR Master Mix (A6002, Promega, Madison, WI) in QuantStudion 6 Flex Real-Time PCR System. The PCR program was as follows: 95 °C for 2 min, and then followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s), and the sequence of primers were shown in Table S1.

### Measurement of Cytochrome P450 (CYP1A1) Activity

The activity of CYP1A1 was measured by the P450-Glo assays luminescent assay kit (V8752, Promega). Astrocytes were seeded 96-well for 24 h. According to the manufacturer's protocol of the GloMax-Multi+ Detection System (E8032, Promega), luminescence was measured in each well. Meanwhile, cell number was evaluated by using CellTiter-Glo Assay (G7570, Promega) in each well. To compensate for variability in cell number, the CYP1A1 activity in per cell was calculated from that measured luminescence values divided the cell number. For evaluating group difference, the TCDD groups of CYP1A1 activity in per cell were normalized by dividing DMSO group (Control group).

### Autonomous Motility Analysis

The astrocytes were cultured in 96 Cell Carrier Ultra (PerkinElmer, Waltham, MA). When cell reached 80% confluency, astrocytes were incubated with 0.01, 0.03, and 0.1 nmol/L TCDD or solvent (DMSO) in the absence or presence of the 10 μmol/L CH223191 in low serum medium for 3 h. Hoechst 33342 (R37605, Invitrogen, Carlsbad, CA) was used to stain the nucleus for cell number calculation. The astrocytes were incubated in a live cell chamber with 5% $CO_2$ at 37 °C. Individual astrocyte was tracked with Opera Phenix High content screening system at 30 min intervals within 48 h. The total live cell images were analyzed via Harmony software (version 4.8, PerkinElmer). Astrocyte displacement represented represent the straight distance between the start point and the end point of cell movement was quantified at 12, 24, 36, and 48 h, including the accumulated distance and displacement. The accumulated distance is the total distance of cell movement from the start to the end points, the displacement shows the straight-line distance of cell movement from the start to the end points.[19]

### Enzyme-Linked Immunosorbent (ELISA) Assay

Astrocytes were seeded in 6-well plates, then were treated with 0.03 nmol/L TCDD or 0.1% DMSO. After 12, 24, 36, 48, 72, and 96 h, the medium supernatant was collected and the concentration of Cxcl2, Ccl7, and Cxcl10 were measured by ELISA kits. According to the manufacturer's protocol, the samples and serially diluted standards were separately added to wells precoated anti-Cxcl2, anti-Ccl7, or anti-Cxcl10 antibody. After 1∼2 h incubation at room temperature with gentle shaking, each well was washed for 4 times with wash buffer. Biotinylated antibody of Cxcl2 or Cxcl10 was added afterward and incubated at room temperature with gentle shaking. Then each well was washed with wash buffer 4 times, and Cxcl2 Streptavidin solution/Ccl7 TMB Substrate/Cxcl10 Streptavidin-HRP-substrate was added and incubated for 10−30 min at room temperature with gentle shaking. Stop solution was added before signal detection at 450 nm. The results were analyzed by a four-parameter curve fit.

### Western Blot Analysis

Primary cultured neurons were treated with conditional medium from the DMSO/TCDD-treated astrocytes, and then were lysed with cell extraction buffer (FNN0011, Invitrogen). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in Odyssey Blocking Buffer (927−40000, LI-COR, Lincoln, NE) for 3 h and incubated with 1:1000 anti-Synaptotagmin-1, anti- PSD95 or 1:10000 anti-β-actin antibody overnight at 4 °C (A2228, Sigma). After washes in PBST (phosphate buffered solution with 0.05% Tween 20), membrane was incubated in


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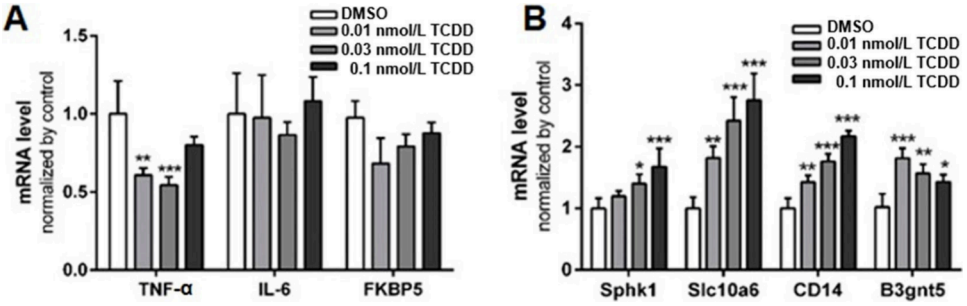


**Figure 1.** Changes in expression levels of reactive astrocyte marker genes after TCDD treatment. Astrocytes treated with TCDD (0.01, 0.03, 0.1 nmol/L) or 0.1% DMSO (control) for 24 h. The mRNA expression of (A) A1 reactive astrocytes (TNF-$\alpha$, IL-6, FKBP5) and (B) A2 reactive astrocytes (Sphk1, Slc10a6, CD14, and B3gnt5) markers were analyzed by qPCR. GAPDH was used as an internal control. Values were folds of control and expressed as mean ± SEM ($n$ = 3), and each independent sample was detected in triplicate. Statistical analysis was done by one-way ANOVA with Bonferroni test. Statistically significant difference *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001 compared with the DMSO groups.

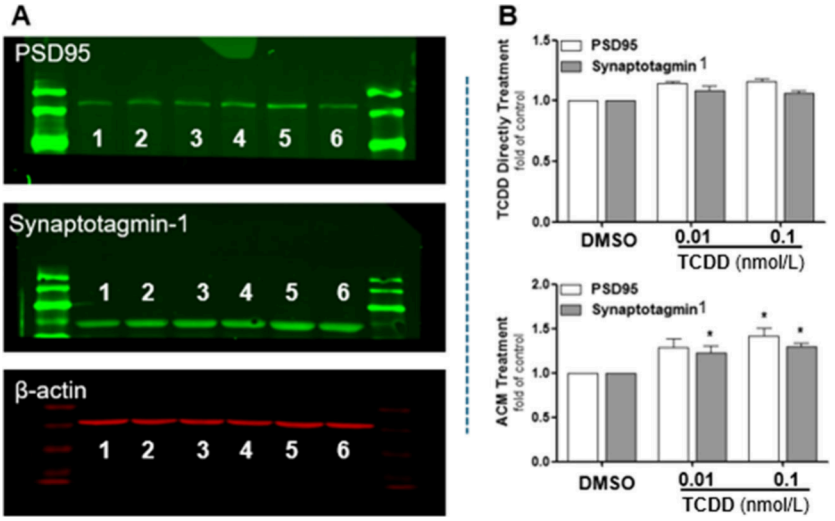


**Figure 2.** Enhanced protein expression of PSD95 and Synaptotagmin-1 in neurons treated with conditional medium from TCDD-induced A2-type astrocytes. (A) The bands 1−3 in sequence were neurons (3 days *in vitro*) treated with the directly mixture of medium and 0.1% DMSO, 0.01, 0.1 nmol/L TCDD for 4 days (TDM group), and bands 4−6 in sequence were neurons treated with the mixture of astrocytic conditional medium after 0.1% DMSO, 0.01, 0.1 nmol/L TCDD treatment (TACM group). Western blotting analysis of PSD95 and Synaptotagmin-1 in whole-cell extracts prepared from the cultured neurons. The protein was visualized using an Odyssey CLx Infrared Imaging System (Li-COR), with $\beta$-actin as a loading control. One of the representative images was shown, $n$ = 3. (B) Protein amount of PSD95 and Synaptotagmin-1 were quantified and normalized by the loading control. Values were fold of corresponding control group (DMSO) and expressed as mean ± SEM ($n$ = 3). Each independent sample was detected in triplicate. Statistical analysis was done by one-way ANOVA with Bonferroni test. Statistically significant difference *$p$ < 0.05 compared with the corresponding DMSO groups.

Goat anti-Rabbit lgG (H+L) second antibody (926-68073, LI-COR) or Goat anti-Mouse lgG (H+L) second antibody (926-32210, LI-COR) for 1 h at room temperature with gentle shake. Signals were collected by Odyssey infrared imaging (LI-COR) and analyzed with Image Studio Lite, Ver. 5.2.

### Statistical Analysis and Other Assays

GraphPad prism software (version 5.0, La Jolla, CA) was used to statistical analysis and plotting figures. Statistical tests were done by using Student's $t$ test, one-way ANOVA, or two-way ANOVA. Statistically significant changes were classed as [*] where $p$ < 0.05, [**] where $p$ < 0.01, and [***] where $p$ < 0.001.

## RESULTS AND DISCUSSION

### Environmental Related Doses TCDD Led to A2 Type Astrocytic Activation

To reveal the effects of TCDD exposure on astrocytic activation, we examined the expression of A1 type marker genes (TNF-$\alpha$, IL-6 and FKBP5) and A2 type marker genes (Sphk1, Slc10a6, CD14, and B39d5) in astrocytes[7] upon TCDD treatments. The results showed that 0.01 and 0.03 nmol/L TCDD reduced the TNF-$\alpha$ mRNA level (39.2% and 45.5%, respectively) (Figure 1A). While 0.01 to 0.1 nmol/L TCDD treatment increased the expression of A2 reactive marker genes in a dose-dependent manner, including Sphk1, Slc10a6, and CD14 (Figure 1B). To further evaluate the function of TCDD-activated astrocytes on the nervous system, we investigated the effects of a conditioning medium derived from TCDD-treated astrocytes on primary cultured cortical neurons. The neurons were cultured in direct medium (DM) supplemented with DMSO or TCDD (0.01 and 0.1 nmol/L, TDM groups) or in conditioned medium collected from astrocyte cultures (ACM) after treatments with DMSO or TCDD (0.01 and 0.1 nmol/L, TACM groups) for 4 days. We found that the expression of synaptotagmin-1 and PSD95 protein were significantly increased in TACM group, but not in TDM group (Figure 2A and B). It indicated that the conditioned medium from the 0.01 and 0.1 nmol/L TCDD-treated astrocytes exhibited synaptic promoting effects on the


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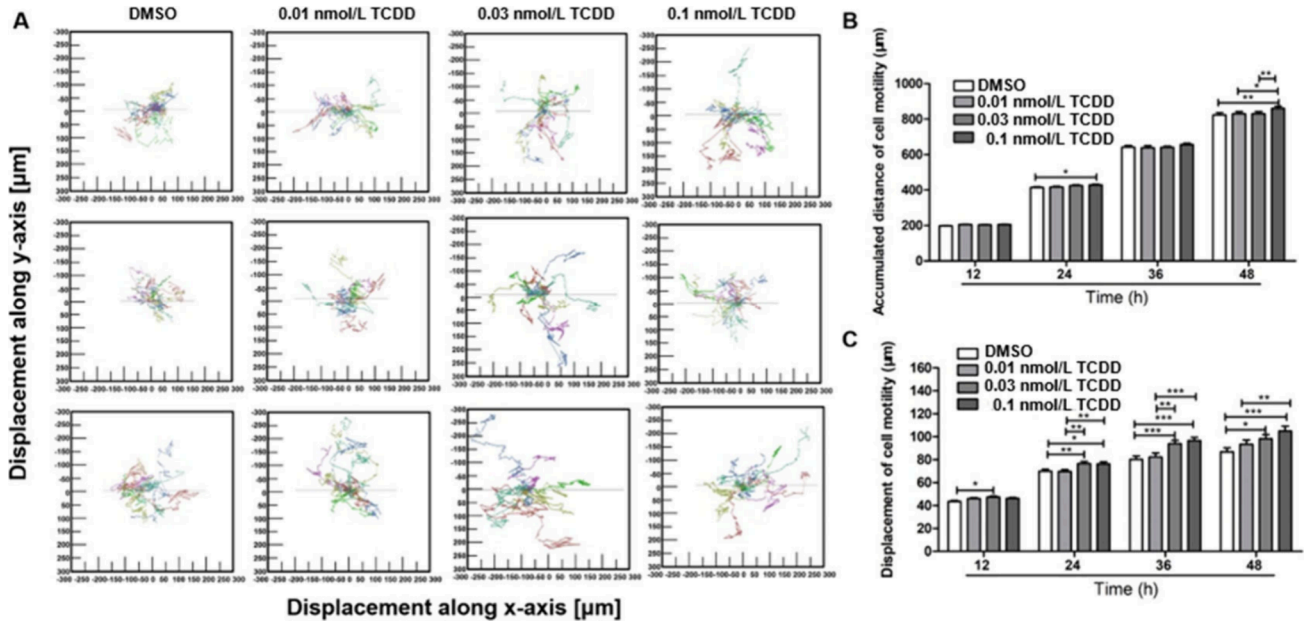


**Figure 3.** Effects of TCDD on autonomous motility of astrocytes. Astrocytes were treated with TCDD (0.01, 0.03, or 0.1 nmol/L) or 0.1% DMSO. (A) Representative trajectories of tracked astrocyte for 48 h. Each line showed the movement route of single cell within 48 h time period. Each image contains ten tracked cells from three randomly selected fields in each well of 96-well-plate ($n$ = 10). The starting positions of tracks were adjusted to the same point of the coordinate axis according to the actual movement distances. (B) Accumulated distance of astrocyte at 12, 24, 36, and 48 h. (C) Displacement of astrocyte at 12, 24, 36, and 48 h. Values were expressed as mean ± SEM ($n$ > 208) and each independent sample was detected in sextuple. Statistical analysis was done by two-way ANOVA with Bonferroni test. Statistically significant difference *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001 compared with the DMSO groups (12 h).

cortical neurons, which supported the astrocytic response to low-dose TCDD as the protective A2 type. The A2 type astrocytes have protective effects by promoting synaptic formation and maturation. Under ischemia conditions, A2-type astrocytes were induced to promote recovery and repair by proliferation and supporting neuronal regeneration.[20,21]

However, higher doses of TCDD led to a complex cellular response. In Figure S1, 10 nmol/L TCDD can induce a significant increase in mRNA expression of both A1 type marker genes (TNF-$\alpha$, IL-6, IL-1$\beta$) and A2 type marker genes (Slc10a6, CD14, B3gnt5). Some A2 type marker genes (Slc10a6, B3gnt5) were also upregulated under 100 nmol/L TCDD exposure. The results showed that high concentrations of TCDD led to a combination of both A1 and A2 type astrocytic activation. Several in vivo and in vitro studies have demonstrated that 10 nmol/L TCDD exposure could induce the expression and secretion of TNF-$\alpha$, an A1 type marker gene.[6,22,23] A1/A2 type astrocytes could be transformed into each other under certain stimulation[24], whether there is a transformation between different types of TCDD induced astrocyte activation still needs to be clarified. A1 type astrocytes are usually induced after acute central nervous system (CNS) injury and present in brain regions of neurodegenerative diseases. With a loss of main astrocyte functions, A1-type astrocytes secreted neurotoxin to induce the death of neurons and oligodendrocytes.[1] Taken together, relatively high doses TCDD exposure might lead to A1 type astrocytic activation which could be harmful to the interacting neurons. It suggests that whether astrocytes have protective or damaging effect on the nervous system depends on the concentration of pollutants.

## TCDD Promoted Autonomous Motility of the Astrocytes

Enhancement in cell motility is one of the cellular events of reactive astrocytes. We employed a newly established high content analysis-based real-time living cell recording system[19] to track the autonomous motility of the cultured astrocytes. Astrocytes incubated with TCDD (0.01 or 0.03 nmol/L) or DMSO were tracked alive for 48 h. By adjusting initial positions of all tracked cells to the same origin point of the coordinate axis, a compilation of the tracks corresponding to single astrocyte migration paths intuitively showed that TCDD treatment groups had a wider range of motion than control at 48 h (Figure 3A). We found that, compared with the control group, the 0.1 nmol/L TCDD treatment group significantly increased the cumulative trajectory distance of astrocytes by about 36.3 $\mu$m after 48 h of treatment (Figure 3B). Consistent with the track results, TCDD treatment significantly promoted the displacement of cell motility in a time-dependent manner (Figure 3C). In our previous work, we reported 1 nmol/L TCDD exposure could promote the migration ability of astrocyte in wound-healing assay and Transwell assay;[13] in both of which, there are man-made triggers that cause cell to migrate, such as scratch or gravity. With the development of high content screening technology, it has become possible to study the changes in cellular autonomous movement. In this study, we obtained consistent results with lower doses. These results indicated that TCDD activates astrocytes and promotes their autonomous movement.

## TCDD Induced the Expression and Secretion of Cxcl2, Ccl7, and Cxcl10 in Astrocytes

To gain further insight into the mechanisms of TCDD-induced astrocytic activation, microarray was employed to study the differentially expressed genes in the astrocytes in response to 96 h TCDD treatment (0.1 nmol/L). Cytokine−cytokine


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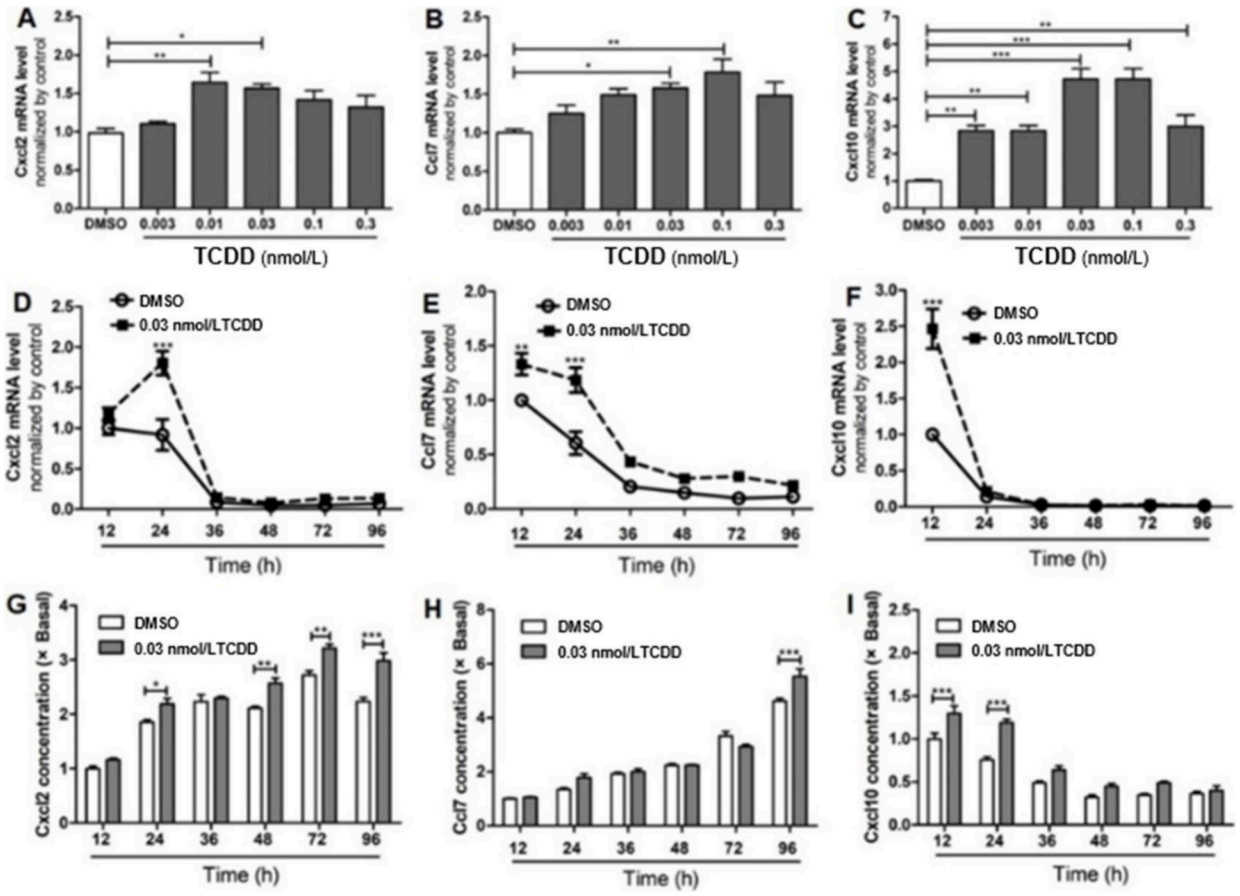


**Figure 4.** Effects of TCDD on expression and secretion of chemokines in astrocytes. Cells were incubated with different concentrations of TCDD (0.03, 0.01, 0.03, 0.1, or 0.3 nmol/L) or 0.1% DMSO for 24 h. The mRNA expression levels of (A) Cxcl2, (B) Ccl7, and (C) Cxcl10 were determined by qPCR in dose-dependent effects of TCDD for 24 h. Cells were treated with 0.03 nmol/L TCDD or 0.1% DMSO for 12, 24, 36, 48, 72, or 96 h. The mRNA expression levels of (D) Cxcl2, (E) Ccl7, and (F) Cxcl10 were determined by qPCR in TCDD time-dependent effects. GAPDH was an internal control for quantification. The protein expression of (G) Cxcl2, (H) Ccl7, and (I) Cxcl10 were determined by ELISA. Data are shown as fold of control and expressed as mean ± SEM ($n = 3$) and each independent sample was detected in triplicate. Statistical analysis was done by one-way ANOVA with Bonferroni test in dose-dependent effects of TCDD for 24 h or two-way ANOVA with Bonferroni test in TCDD time-dependent effects. Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the DMSO groups.

receptor interaction and chemokine signaling pathway were enriched in a high proportion by KEGG enrichment analysis (Figure S2). We screened the expression of a series of chemokine-encoding genes by qPCR analysis in enriched pathways, which were related to cell motility (Figure S3). There were significant differences in the changes of Cxcl2, Ccl7, and Cxcl10 mRNA after TCDD treatment. After exposure to 0.01 and 0.03 nmol/L TCDD, the mRNA expression of Cxcl2 was induced to increase by 64.5% and 56.8%, respectively (Figure 4A). While the mRNA expression of Ccl7 was increased by about 57.5% and 78.5% in 0.03 and 0.1 nmol/L TCDD groups, respectively (Figure 4B). TCDD also significantly upregulated Cxcl10 mRNA expression. Compared with the other two chemokines, the range of the effective TCDD concentrations was wider (0.003−0.3 nmol/L), suggesting Cxcl10 more sensitive in response to TCDD (Figure 4C). Based on the data of the time-course study, we found that the basal expression level of the tested chemokines in the DMSO groups decreased over time. Particularly, the gene expression levels were such low in cells with drug treatments longer than 36 h (Figure 4D−F). TCDD (0.03 nmol/L) increased Cxcl2 mRNA expression upon 12 and 24 h treatments with a higher effect at 24 h (Figure 4D). The mRNA of Cxcl10 reached a peak expression after 12 h TCDD treatment and then dropped to the minimum level (Figure 4F). Unlike Cxcl10 or Cxcl2, the inductive effect of TCDD on Ccl7 mRNA expression lasted longer (Figure 4E). In addition, the protein secretion levels of chemokines in astrocyte culture medium were detected by ELISA, and it was found that TCDD treatment induced the secretion of Cxcl2 protein in a time-dependent manner, with a secretion amount of approximately 1.3−1.8 times that of the control group (Figure 4G). Similarly, after 96 h of treatment with TCDD, the secretion of Ccl7 protein increased by 20% (Figure 4H). However, TCDD only significantly and briefly increased the secretion of Cxcl10 by 1.3 and 1.4 times at time points 12 and 24 h, respectively, before gradually returning to similar levels as the control group (Figure 4I).

Astrocytes initiate cell-to-cell communication primarily by producing cytokines or chemokines. Three types of chemokines, Cxcl10, Cxcl2, and Ccl7 were characterized in the conditional medium from 0.1 nmol/L TCDD-treated astrocytes. After TCDD treatments, the Cxcl10 mRNA level (known as interferon-gamma inducible protein 10 or IP10) was rapidly increased within 12 h and the protein of Cxcl10 secreted in the medium was significantly increased within 24 h.


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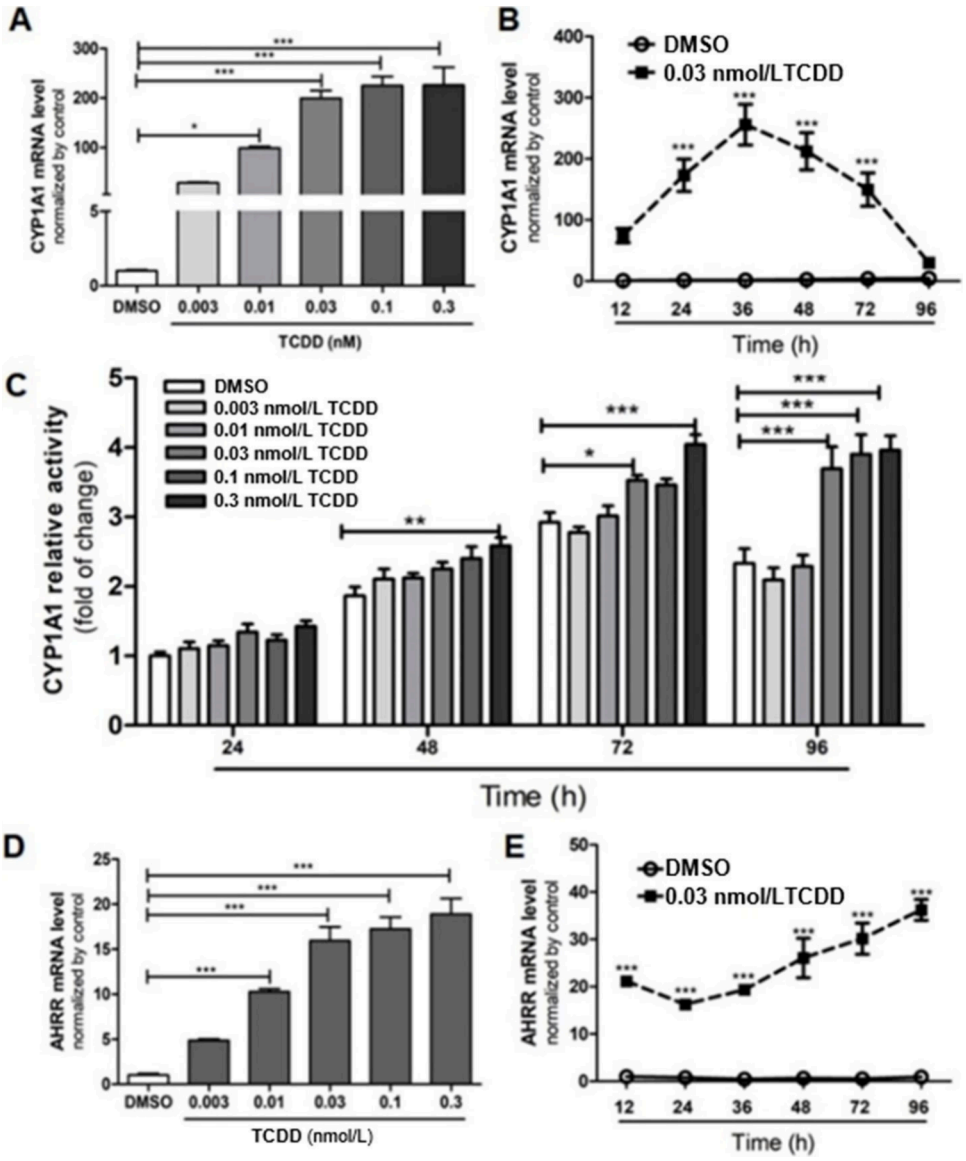


**Figure 5.** Roles of AhR pathway in mediating the changes in the reactive astrocytes induced by TCDD. (A) Astrocytes were treated with TCDD (0.003−0.3 nmol/L) or 0.1% DMSO for 24 h. The mRNA expressions of CYP1A1 were determined by qPCR analysis. (B) After 12, 24, 36, 48, 72, or 96 h treatment with 0.03 nmol/L TCDD or 0.1% DMSO, the mRNA levels of CYP1A1 were determined by qPCR analysis. (C) Quantification of CYP1A1 luminescence was determined by P450-Glo assays. (D) Dose- or (E) time-dependent effect of AHRR gene expressions were determined by qPCR analysis. Data were shown as fold of control and expressed as mean ± SEM ($n$ = 3) and each independent sample was detected in triplicate. Statistical analysis was done by one-way ANOVA with Bonferroni test in dose-dependent effects of TCDD for 24 h or two-way ANOVA with Bonferroni test in TCDD time-dependent effects. Statistically significant difference *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001 compared with the DMSO groups.

Cxcl10 is not only an activation marker for astrocytes,[25] Tanuma et al. found that Cxcl10 secreted by astrocytes can activate astrocytes in an autocrine or paracrine way, leading to direct reactive glial cell proliferation and subsequent migration and activation of microglia/macrophages.[26] Lee et al. also reported that the astrocyte-derived Cxcl10 played a central role in LCN2-induced cell migration of microglia and neurons, as well as astrocytes themselves.[27] Thus, the present result that TCDD increased the autonomous motility of astrocytes might be a subsequent consequence of the Cxcl10 induction. Unlike Cxcl10, the induction of Cxcl2 and Ccl7 by TCDD lasted relatively longer to 96 h, which might contribute to the upregulation of the marker proteins (Synaptotagmin-1 and PSD95) of synaptic formation and maturation in conditional medium-treated cortical neurons. It has been found that the inductions of Cxcl2 and Ccl5 by the hepatocyte growth factor significantly increase axon outgrowth in rat primary hippocampal neurons.[28] And Ccl7 could promote the differentiation of dopaminergic precursors into dopaminergic neurons and the maturation of dopaminergic neurons.[29]

## TCDD Induces the Activation of Astrocytes in an AhR-Dependent Manner

To clarify the mediating role of AhR in TCDD induced activation of astrocytes, we investigated the activation of the AhR signaling pathway, particularly at the effective TCDD concentrations mentioned above. We found that downstream marker genes of AhR (including CYP1A1 and AHRR) were upregulated after TCDD treatment, indicating activation of the


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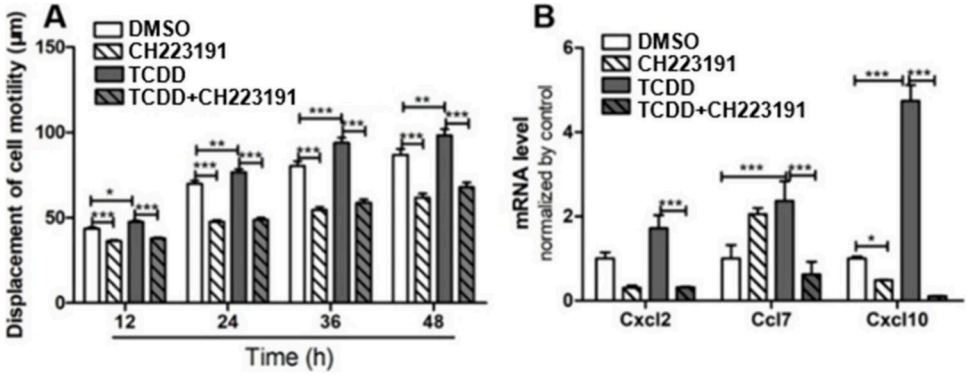


**Figure 6.** Roles of AhR pathway in the effects of TCDD on autonomous motility and the expression of chemokines of the astrocytes. Astrocytes treated with TCDD (0.03 nmol/L) or 0.1% solvent control together with or without CH223191 (1 $\mu$mol/L) were continuously tracked for 48 h. (A) Displacement of cell motility was collected separately at 12, 24, 36, and 48 h. Values were expressed as mean ± SEM ($n \geq 208$) and each independent sample was detected in sextuple. (B) Cxcl10, Cxcl2 and Ccl7 mRNA level in cultured astrocytes were inhibited by CH223191 incubated with 0.03 nmol/L TCDD or 0.1% DMSO for 24 h. Data were shown as fold of control and expressed as mean ± SEM ($n = 3$) and each independent sample was detected in triplicate. Statistical analysis was done by two-way ANOVA with Bonferroni test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control.

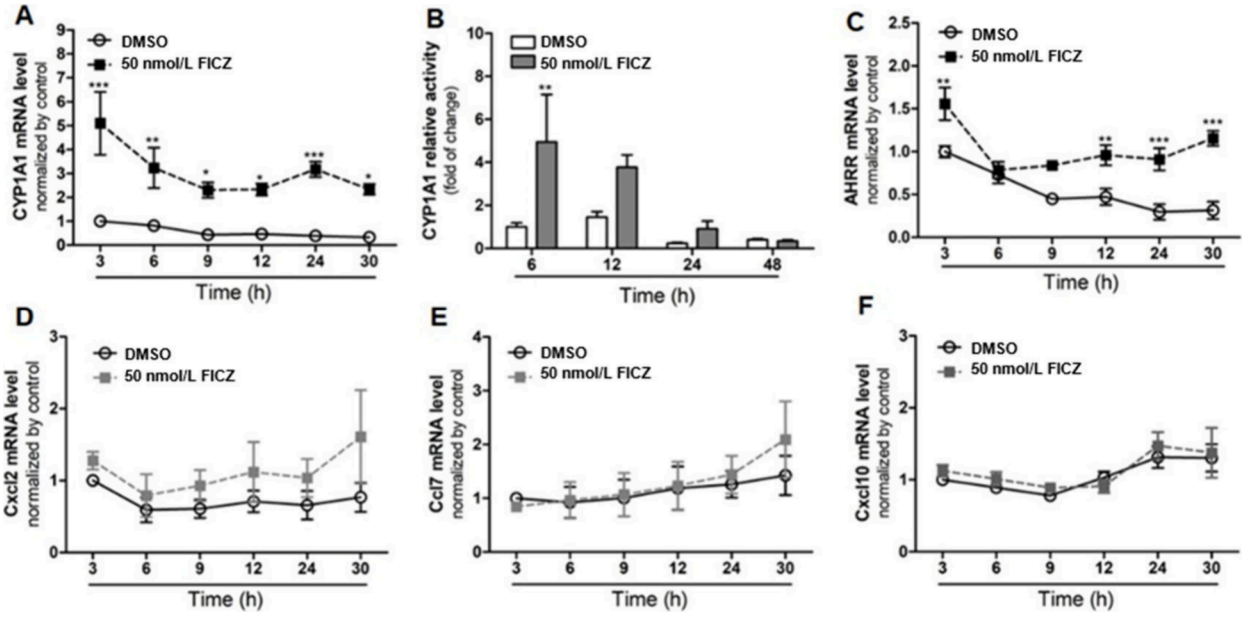


**Figure 7.** Effects of FICZ on the expression of AhR downstream genes and chemokines in astrocytes. After 3, 6, 9, 12, 24, or 30 h treatment with FICZ (50 nmol/L) or 0.1% DMSO. The mRNA level of (A) CYP1A1, (C) AHRR, (D) Cxcl2, (E) Ccl7, and (F) Cxcl10 were determined via qPCR. GAPDH was used as an internal control. (B) CYP1A1 activity of astrocytes was measured by P450-Glo Assays. Values were shown as fold of control (DMSO group) and expressed as mean ± SEM ($n = 3$) and each independent sample was detected in triplicate. Statistical analysis was done by two-way ANOVA with Bonferroni test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control.

AhR pathway (Figure 5). The mRNA expression of CYP1A1 was induced in a concentration-dependent manner after the TCDD treatment, and the maximum induction was 200-fold of control (Figure 5A). The time course results showed that the inducive effect of TCDD on CYP1A1 mRNA reached its maximum value at 36 h and gradually decreased after that (Figure 5B). In line with the mRNA expression, the enzyme activity of CYP1A1 was increased in concentration- and time-dependent manners after the TCDD treatment (Figure 5C). The mRNA expression of AHRR was also induced in dose- and time-dependent manners after the TCDD treatment (Figure 5D and E). Different from that of CYP1A1, the induction effect of TCDD on AHRR mRNA expression was persistent throughout the entire exposure period (Figure 5E).

The specific antagonist CH223191 of AhR was used to further validate the role of AhR in the TCDD-induced reactive astrocytes. With the pretreatment of CH223191 (Figure S4), we found that the displacement of the autonomous astrocyte motility was significantly decreased compared with TCDD treatment alone (Figure 6A). Pretreatment with CH223191 alone can significantly inhibit the autonomous movement displacement of astrocytes at different time points. Meanwhile, CH223919 pretreatment remarkably suppressed the mRNA expressions of chemokine (Cxcl2, Ccl7, and Cxcl10) induced by TCDD (Figure 6B). Especially, the CH223191-treated alone group significantly inhibited Cxcl10 mRNA expression. Therefore, blocking AhR alleviated the phenomenon of TCDD activating astrocyte cells and gene expression.


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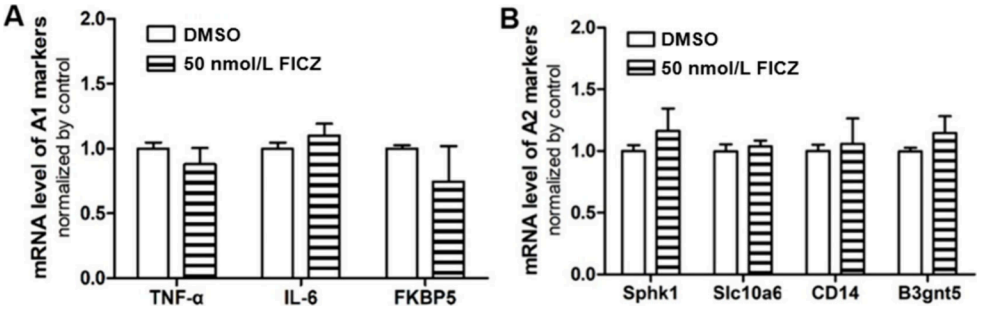


**Figure 8.** Effects of FICZ on the expression of astrocytic activation marker genes. Astrocytes treated with 50 nmol/L FICZ or 0.1% DMSO (control) for 12 h. The mRNA expression of (A) A1 type marker genes and (B) A2 type marker genes were analyzed by qPCR. GAPDH was used as an internal control. Values were fold of controls and expressed as mean ± SEM ($n$ = 3) and each independent sample was detected in triplicate. Statistical analysis was done by one-way ANOVA with Bonferroni test. Statistically significant difference $^{**}p < 0.01$ compared with the DMSO groups.

AhR is the most important receptor of dioxins mediating the toxic effects. This study found that simultaneous with astrocytic activation, the expression of classical AhR downstream marker genes, CYP1A1 and AHRR, were drastically up-regulated in response to 0.01−0.03 nmol/L TCDD treatments. These results indicated that in TCDD-induced reactive astrocytes, the AhR pathway was normally activated. Furthermore, the TCDD-induced Cxcl10, Cxcl2, and Ccl7 up-regulation and autonomous motility enhancement were decreased when AhR was blocked by its antagonist, supporting the notion that the TCDD-induced astrocytic activation is AhR-dependent. It is generally agreed that AhR binds to the dioxin-responsive elements (DREs) located upstream of target gene promoters to regulate gene expression.[30] Promoter sequence analysis revealed one DRE consensus sequence at the −168 upstream of Cxcl2 promoter, and three DREs at the −2509, −2472, −1448 upstream of Cxcl10 promoter.[31] Thus, the TCDD-induced activated AhR might functionally bound to one or more DRE elements at the promoters to directly regulate the transcription of the chemokines.

### FICZ Activated AhR Pathway in Astrocyte but Not Changing the Expression of Astrocytic Activation Marker Genes and Chemokine Genes

The effects of the endogenous AhR ligand FICZ on the astrocytic activation were investigated. We found that 50 nmol/L FICZ[32] induced CYP1A1 mRNA expression to the peak as early as 3 h, and then in a declined trend, but still higher than that of control (Figure 7A). The maximum induction was 5-fold of control group (Figure 7A). The enzymatic activity of CYP1A1 was induced similarly with that of CYP1A1 mRNA upon FICZ treatment (Figure 7B). The FICZ-induced up-regulation of AHRR gene was decreased first and then increased slowly (Figure 7C). Subsequently, we found that FICZ only subtly and without preference altered a few A1 and A2 marker genes, which has no statistical significance (Figure 8). Meanwhile, no obvious change of Cxcl10, Cxcl2, and Ccl7 mRNA expression was observed in astrocytes after FICZ treatment (Figure 7D−F).

Given the ligand diversity in efficiency and efficacy of AhR activation and downstream effects,[33] we further addressed whether the AhR-dependent A2 type activation of astrocytes is TCDD-specific. FICZ is a well-known tryptophan photoproduct activating the AhR, which could activate AhR pathway in astrocytes. In this study, FICZ could alter the expression of several activation markers, but with no A2-type preference. Moreover, the TCDD-sensitive chemokines were not responsive to FICZ treatment. Thus, different effects of AhR-mediated gene regulation on astrocyte activation-related gene regulation were found between TCDD and FICZ. Several studies also reported different biological and toxicological responses between FICZ and TCDD. Higher doses TCDD induced nongenomic responses led to rapid TAK1 and NF-κB p65 phosphorylation, resulting in the release of TNF-α and astrocyte activation and then the initiation of neuronal apoptosis.[22] However, as an AhR agonist, FICZ suppressed the activation of NF-κB p65 induced by TNF-α and then the inflammatory effect was reduced.[34] Thus, the "nongenomic" AhR-dependent mechanisms (such as protein phosphorylation and ubiquitination) might partially contribute to the different results from the different AhR ligands treatment. Similar diversity has also been found in the study of T cell differentiation. TCDD and FICZ have divergent effects on delayed-type hypersensitivity response and T cell differentiation, which was mediated through, at least in part, regulation of miRNA-132.[35] In our study, the effect of TCDD and FICZ on AhR activation might be different, which has been found in other cells, that TCDD induced the expression of CYP1A1 up to 270-fold, which FICZ was 34-fold.[36] However, the molecular mechanisms underlining the divergent effects of TCDD and FICZ still need further investigation, such as the involvement of epigenetic mechanisms.

## CONCLUSION

In this study, we demonstrated that environment-relevant doses TCDD exposure induced A2-type of astrocytic activation with enhanced autonomous motility and up-regulation of chemokines via AhR pathway. TCDD-induced A2 type astrocytes might promote the synaptic formation and maturation of neurons. Both the two AhR ligands (TCDD and FICZ) could activate the AhR pathway, but the endogenous AhR ligand, FICZ neither led to astrocytic activation, nor affected the expression of the chemokines induced by TCDD.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00189.

Isolation and culture methods for astrocyte and neuron, Table S1, and Figures S1−S5 (PDF)



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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DMSO, dimethyl sulfoxide; AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-*b*] carbazole; CNS, central nervous system; DLCs, dioxin-like compounds; AhRR, AhR repressor; MEM, minimum essential medium; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified Eagle medium; qPCR, quantitative real-time PCR; PBST, phosphate buffered solution with 0.05% Tween 20; DM, direct medium; TDM groups, direct medium supplemented with DMSO or TCDD; ACM, conditioned medium collected from astrocyte cultures; TACM groups, conditioned medium collected from astrocyte cultures after treatments with DMSO or TCDD

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