Toxicology Letters 184 (2009) 90–96

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Toluene disrupts synaptogenesis in cultured hippocampal neurons

Huei-Min Lin[a,1], Chih-Yang Liu[b,1], Guey-Mei Jow[c], Chih-Yung Tang[a,*]

[a] Department of Physiology, College of Medicine, National Taiwan University, Taipei, Taiwan
[b] Department of Health, Taoyuan General Hospital, Taoyuan, Taiwan
[c] School of Medicine, Fu Jen Catholic University, Hsin-Chuang, Taipei County, Taiwan

ARTICLE INFO

Article history:
Received 12 August 2008
Received in revised form 22 October 2008
Accepted 26 October 2008
Available online 7 November 2008

Keywords:
Neurotoxicology
Synaptogenesis
Confocal microscopy
Glutamatergic synpases
Immunofluorescence

ABSTRACT

Prenatal toluene exposure may lead to significant developmental neurotoxicity known as fetal solvent syndrome. Emerging evidence suggests that toluene embryopathy may arise from an elusive deviation of the neurogenesis process. One key event during neural development is synaptogenesis, which is essential for the progression of neuronal differentiation and the establishment of neuronal network. We therefore aim to test the hypothesis that toluene may interfere with synaptogenesis by applying toluene to cultured hippocampal neurons dissected from embryonic rat brains. In the presence of toluene, hippocampal neurons displayed a significant loss of the immunostaining of synapsin and densin-180 punctas. Notably, a dramatic reduction was also discerned for the colocalization of the two synaptic markers. Moreover, Western blotting analyses revealed that toluene exposure resulted in considerable down-regulation of the expression of synapse-specific proteins. None of the preceding observations can be attributed to toluene-induced cell death effects, since toluene treatments failed to affect the viability of hippocampal neurons. Overall, our data are consistent with the idea that toluene may alter the expression and localization of essential synaptic proteins, thereby leading to a disruption of synapse formation and maintenance.

1. Introduction

Toluene is a volatile aromatic compound widely used in the industry as an organic solvent and can be found in many household products such as adhesives, cleaning agents, and paint thinners (Low et al., 1988). Like some other volatile organic compounds, however, toluene has also been inappropriately exploited as an abused inhalant that has become a serious and potentially fatal problem among young people (Anderson and Loomis, 2003). Long-term recreational, occupational, as well as environmental exposure to toluene may result in a variety of neurological manifestations, including ataxia, dementia, sensory dysfunction, seizure, and tremor (Benignus, 1981; Anderson and Loomis, 2003). Furthermore, emerging evidence suggests that women who abused toluene during pregnancy are prone to give birth to infants with congenital neurological defects such as developmental delays, microcephaly, and cognitive impairments (Arnold et al., 1994; Jones and Balster, 1998). This raises the possibility that prenatal toluene exposure may lead to significant embryopathy, which is also known as fetal solvent syndrome (Jones and Balster, 1998; Costa et al., 2002).

The exact mechanism underlying toluene embryopathy remains elusive. Although a few animal models for toluene embryopathy were able to demonstrate significant postnatal neurobehavioral deficits in the offspring of pregnant dams chronically treated with toluene (Hass et al., 1999; Hougaard et al., 1999), a clear and consistent neuropathological change in the brain was rarely found in young rats subjected to prenatal toluene exposure (Gospe et al., 1994; Thiel and Chahoud, 1997; Dalgaard et al., 2001). The most prominent effect reported was a reduction of the number of cortical neurons in response to prenatal toluene exposure, although the brain weight was still within normal range, suggesting that toluene may induce alterations in neurogenesis and neuronal migration (Gospe and Zhou, 2000). These observations imply that the neurobehavioral deficits of toluene embryopathy may arise from a subtle defect during the neural development process that leads to a long-term deficiency in the structure and function of the brain.

One key developmental event with which toluene may interfere is synaptogenesis. Synaptogenesis in the central nervous system begins early in the embryo and extends well into postnatal life; moreover, synaptogenesis is closely associated with the establishment of neuronal differentiation and neuronal network, and is thus essential for the precision of brain circuitry (Benson et al., 2001). Since synaptogenesis is a long-term process that involves multiple

* Corresponding author at: Department of Physiology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., Sec. 1, 10F, Taipei 100, Taiwan.
Tel.: +886 2 23562215; fax: +886 2 23964350.
E-mail address: tang@ntu.edu.tw (C.-Y. Tang).
[1] These authors contributed equally to this work.

0378-4274/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2008.10.027

stages such as axon guidance, synapse formation, synapse maintenance (stabilization), and activity-dependent synapse elimination (Cohen-Cory, 2002; Vicario-Abejon et al., 2002), a mild disruption imposed by toluene may produce considerable impact on subsequent progressions of neural development. The aim of this study is therefore to test the hypothesis that toluene may affect synaptogenesis. By use of cultured hippocampal neurons dissected from embryonic rat brains, we present immunofluorescence and biochemical evidence suggesting that prenatal toluene exposure yields significant perturbation of both synapse formation and maintenance.

2. Materials and methods

2.1. Animals

15-Day pregnant Sprague–Dawley (SD) rats were purchased from BioLasco (Taipei, Taiwan) and individually kept in a single cage in the in-house maintenance facility for 3 days before being sacrificed. All animals were handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All procedures involving animals were performed in conformity with the animal protocol approved by the Lab Animal Council, National Taiwan University.

2.2. Hippocampal cultures

Dissociated hippocampal cultures were prepared from SD rats, using a previously described protocol (Banker and Goslin, 1998) with a minor modification. In brief, hippocampi were dissected from the brains of embryonic day 18 embryos, whose brains were removed and placed in Hank's balanced salt solution containing 10 mM HEPES (pH 7.4) and 1 mM sodium pyruvate. The hippocampus was dissected out and dissociated by incubation with 0.25% trypsin solution. The dissociated cells were plated either on coverslips for immunofluorescence (at a density of 200 cells/mm^2) or in culture dishes for Western blot analysis (at a density of 800 cells/mm^2). Coverslips and dishes were coated with poly-D-lysine (0.1 mg/ml, Sigma Chemical Co., St. Louis, MO) and laminin (30 μg/ml, Sigma). Cultures were maintained in the Neurobasal media supplemented with B27 (2%) and glutamax I (0.5 mM) (Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2 incubator at 37 °C.

2.3. Toluene treatments

Cultured hippocampal neurons were treated with toluene (HPLC grade 99.8%, Mallinckrodt Baker Inc., KY, USA) from 4 to 6 days in culture (DIV). An aliquot of freshly prepared toluene-containing medium was added to the cultures once daily for 3 consecutive days to achieve a final concentration of 1, 5, or 10 mM.

2.4. Assessment of neuronal viability

To examine the effect of toluene on neuronal viability, we quantified the reduction in the conversion of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble colored formazan in cells (Hansen et al., 1989). After toluene treatments, MTT assay was performed by incubating cells with 0.5 mg/ml MTT for 2 h at 37 °C. Cells were washed and then lysed with DMSO. The assay for the formation of formazan was measured at 550 nm in a 96-microtiter plate. Absolute formazan values were normalized and expressed as a percentage of the values for sham-treated sister cultures (defined as 100%). To avoid data discrepancy arising from technical variations (*e.g.*, the quality of dissection and seeding procedures) among different batches of neuronal cultures, MTT data were pooled from at least three different culture preparations.

2.5. Antibodies

The acquired antibodies used in this study include rabbit anti-synapsin I (BD Pharmingen, San Diego, CA, USA), mouse anti-densin-180 (a kind gift from Dr. Mary Kennedy, Caltech, USA), mouse anti-PSD-95 (Affinity BioReagents, Golden, CO, USA), and mouse anti-β-actin (Sigma) antibodies.

2.6. Immunofluorescence

The coverslips containing hippocampal cultures were rinsed in PBS and then fixed with cold methanol at −20 °C for 20 min. After washing with cold PBS, cells were permeabilized and blocked with a blocking buffer (5% normal goat serum in 20 mM phosphate buffer, pH 7.4, 0.1% (v/v) Triton X-100, and 0.45 M NaCl) for 60 min at 4 °C, followed by incubation with appropriate dilutions of primary antibodies (1:1000 for rabbit anti-synapsin I; 1:500 for anti-densin-180) in the blocking buffer overnight at 4 °C. After three washes with blocking buffer, the coverslips were incubated with goat-anti-mouse antibodies conjugated to Alexa568 or goat anti-rabbit antibodies conjugated to Alexa488 (Invitrogen Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature. Finally, the coverslips were rinsed once in blocking buffer, twice in PBS, and twice in 0.1 M carbonate buffer, pH 9.2, before they were mounted on glass slides in a mounting medium (4% n-propyl gallate, 90% glycerol, 0.1 M carbonate, pH 9.2). The fluorescence images of the fixed cultures were viewed and acquired with a Leica TCS SP2 laser-scanning confocal microscope (Leica, Mannheim, Germany). Image analyses were performed using the Simple PCI 5.3.1 software (Compix Inc., Cranberry Township, PA, USA). Synapsin-positive and densin-positive puncta were counted in the proximal 100 μm segments of dendrites. Each experimental group consisted of 30 neurons, and at least four coverslips of the cultured cells were examined.

2.7. Immunoblotting

Hippocampal neurons were homogenized in a SDS buffer (2% SDS, 62.5 mM Tris, pH 6.8, 5 mM EDTA, 10% glycerol), supplemented with a cocktail of protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN, USA). Lysates were cleared by centrifugation at 14,000 rpm, boiled at 100 °C in the presence of 5% β-mercaptoethanol for 5 min, and stored in aliquots at −80 °C until further usage. Protein concentration was determined, using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins (50 μg) were separated by 7.5% or 12% SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with appropriate primary antibodies (1:1000 dilution for anti-β-actin; 1:5000 dilution for anti-densin-180; 1:5000 for anti-PSD-95, 1:10,000 for anti-synapsin I). Bound antibodies were detected on the blots by using enhanced chemiluminescence system (Pierce, Rockford, IL, USA). Data from 3 to 5 independent experiments were pooled together for quantification analyses by using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The apparent molecular weights of protein bands were calculated from the standard curves based on the mobility of molecular mass standards.

2.8. Statistical analyses

All values were presented as mean ± S.E.M. The significance of the difference between two means was tested using the Student's *t*-test, whereas means from multiple groups were compared using the one-way ANOVA analysis. All statistical analyses were performed with the Origin 7.0 software (Microcal Software, Northampton, MA, USA).

3. Results

3.1. Decrease of synaptic punctas in hippocampal cultures treated with toluene

Cultured 4 DIV hippocampal pyramidal neurons were subjected to toluene treatments for 3 days, and harvested on 7 DIV for immunofluorescence studies. To examine the effect of toluene on the synaptogenesis of glutamatergic synapses, we immunostained hippocampal neurons with antibodies for two synaptic markers, synapsin and densin-180. Synapsin is a synaptic vesicle protein that has been extensively used as a marker for presynaptic terminals (De Camilli et al., 1983), whereas densin-180 is a component of the postsynaptic density and can thus be used as a postsynaptic marker for glutamatergic synapses (Apperson et al., 1996). In control experiments (Fig. 1A), where no toluene was present in the culture medium, both synapsin (Fig. 1A, *left panel*) and densin-180 (Fig. 1A, *middle panel*) immunofluorescence displayed profuse punctuate patterns that labeled the location of the synapses formed in the culture. A significant portion of synapsin and densin-180 immunostaining showed colocalization pattern (Fig. 1A, *right panel*), consistent with idea that these punctas indeed corresponded to glutamatergic synapses. The abundance of synapsin and densin-180 immunofluorescence punctas in cultured neurons, however, was dramatically reduced in response to 5–10 mM toluene exposure (Fig. 1C and D). Quantitative analyses of neuronal immunofluorescence data provided further evidence showing that 5 and 10 mM toluene treatments decreased the density (expressed as clusters/100 μm) of both synapsin and densin-180 immunostaining by about 20% and 35%, respectively (Fig. 2A and B). Moreover, 5 and 10 mM toluene treatments reduced the colocalization of synapsin and densin-180 punctas by about