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Letter

Cell-type-specific and differentiation-status-dependent variations in cytotoxicity of tributyltin in cultured rat cerebral neurons and astrocytes

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ABSTRACT — Tributyltin (TBT) is an organotin used as an anti-fouling agent for fishing nets and ships and it is a widespread environmental contaminant at present. There is an increasing concern about imperceptible but serious adverse effect(s) of exposure to chemicals existing in the environment on various organs and their physiological functions, e.g. brain and mental function. Here, so as to contribute to improvement of and/or advances in *in vitro* cell-based assay systems for evaluating brain-targeted adverse effect of chemicals, we tried to evaluate cell-type-specific and differentiation-status-dependent variations in the cytotoxicity of TBT towards neurons and astrocytes using the four culture systems differing in the relative abundance of these two types of cells; primary neuron culture (> 95% neurons), primary neuron-astrocyte (2 : 1) mix culture, primary astrocyte culture (> 95% astrocytes), and passaged astrocyte culture (100% proliferative astrocytes). Cell viability was measured at 48 hr after exposure to TBT in serum-free medium. IC_{50}'s of TBT were 198 nM in primary neuron culture, 288 nM in primary neuron-astrocyte mix culture, 2001 nM in primary astrocyte culture, and 1989 nM in passaged astrocyte culture. Furthermore, in primary neuron-astrocyte mix culture, vulnerability of neurons cultured along with astrocytes to TBT toxicity was lower than that of neurons cultured purely in primary neuron culture. On the other hand, astrocytes in primary neuron-astrocyte mix culture were considered to be more vulnerable to TBT than those in primary or passaged astrocyte culture. The present study demonstrated variable cytotoxicity of TBT in neural cells depending on the culture condition.

Key words: Rat, Neuron, Astrocyte, Tributyltin, Cytotoxicity

INTRODUCTION

Tributyltin (TBT) is a widespread environmental contaminant, which has been widely used as an agricultural fungicide and an anti-fouling agent for fishing nets and ships. Though these agricultural and fishery applications of TBT have been restricted, TBT has accumulated in mollusks and fish organs to some extent; 27-202 ng/g (fresh mass) in fish muscles and 54-223 ng/g (fresh mass) in fish liver (Shawky and Emons, 1998). Among the Japanese, daily intake of TBT had been estimated to be 2.2-6.9 μg of TBT per day (Tsuda *et al*., 1995). TBT has been reported to have toxic effects on the central nervous system (CNS) as well as immune system (Whalen *et al*., 2002), endocrine system (Hiromori *et al*., 2014), and reproductive system (Adeeko *et al*., 2003) of mammals. Some epidemiological and experimental studies implied possible causal relationships between organotin intoxication and neurological symptoms (Rey *et al*., 1984; Lattanzi *et al*., 2013; Yamada *et al*., 2010). In *in vitro* studies, TBT showed high cytotoxicity in cultured neurons (Yamada *et al*., 2010) and hippocampal slices (Mizuhashi *et al*., 2000), although precise molecular mechanisms of TBT toxicity are largely unknown.

In the neurotoxicology field, primary cultured neural cells (neurons, astrocytes, microglia, and others) are pivotal tools for assessing cytotoxic potencies of chemical compounds as well as for exploring the biological effects

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of these compounds in the CNS. However, there are several fundamental problems to be considered when utilizing these cultures to assess the risk of exposure to a toxic substance in the CNS *in vivo*. First is the type of cells used for the assessment. Usually, pure cultures of one cell type such as pure neuron culture or pure astrocyte culture are preferred or recommended in *in vitro* studies, because results obtained from one specific type of cells are clearer and easier to interpret. However, the CNS is composed of many different types of cells including neurons, astrocytes, microglia, oligodendrocytes, and capillary cells, which interact with one another. In particular, neurons and astrocytes interact closely with each other both directly (e.g. cell adhesion molecules (CAMs)) and indirectly (e.g. via cytokines and gliotransmitters) (Pérez-Alvarez and Araque, 2013; Orellana *et al*., 2013; Ricci *et al*., 2009). Neurons play pivotal roles in CNS function itself, receiving, interpreting, and sending information, and astrocytes support neurons nutritionally and functionally. In other words, the effect of a chemical compound may be significantly modulated by cell-cell interactions among different types of cells in the living CNS. Although it may become more difficult to attribute the observed biological response towards the tested chemical to a specific cell type, mixed cell culture systems would reflect the *in vivo* condition more relevantly than pure cultures of one cell type. The second problem in *in vitro* studies is the difficulty in precisely controlling the differentiation states of cultured cells, due to differences in the developmental stage of the tissue source and/or experimental schedules such as duration of culture.

It is reasonable to assume that the vulnerability of certain cells to chemical exposure depends not only on the cell type but also on the biological state of cells (such as differentiated/undifferentiated, immature/mature, and young/aged) and the existence of other type(s) of cells interacting with them *in vivo*. In the present study, we tried to evaluate cell-type-specific and differentiation-status-dependent variations in the cytotoxicity of TBT in neurons and astrocytes, the two most abundant cells in the CNS, cultured under frequently used cell culture conditions, in which relative abundance of the two cell types are different.

MATERIALS AND METHODS

Animals

Pregnant Wistar ST rats were purchased from SLC (Shizuoka, Japan). They were maintained under controlled conditions (temperature, 24 ± 1°C), on a 12 hr light (06:00-18:00)/12 hr dark (18:00-06:00) cycle. Food and water were freely available. All animal treatments were approved by the Animal Experimentation Committee of Aoyama Gakuin University and were carried out under veterinary supervision and in accordance with the Society for Neuroscience Guidelines for the use of animals in neuroscience research.

Chemicals

Tributyltin hydrochloride (TBT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). TBT was dissolved in DMSO which was used as vehicle in the present study. Final concentration of DMSO in culture medium was 0.01% throughout this study.

Primary neuron culture

Cerebral cortical neurons were isolated from rats at embryonic day 18 (E18). Fetal whole brains were dissected under sterile condition and were transferred to ice-cold isolation medium consisting of equal volumes of Ca^{2+} and Mg^{2+} -free phosphate buffered saline (PBS) and Dulbecco's Modified Eagle's Medium (GIBCO, Palo Alto, CA, USA) supplemented with 10 units/mL penicillin (GIBCO) and 10 μg/mL streptomycin (GIBCO) (DMEM). Subsequently, cerebral cortices were freed of meninges, cut into small pieces, and digested with 2 units/mL papain (Worthington Biochemical Corp., Lakewood, NJ, USA) in PBS containing 0.1 mg/mL DNase (Roche Applide Science, Bavaria, Germany) at 37°C for 30 min. Cells were dissociated gently by passages through a disposable pipette and centrifuged two times in DMEM supplemented with 5% FBS (DMEM/5%FBS) at 800 rpm for 5 min. The cells were resuspended in DMEM/5%FBS and plated onto appropriate culture dishes coated with poly-ethylene-imine (Sigma-Aldrich) at 3,000 cells/mm^2. All cultures were maintained at 37°C in 95% humidified air and 5% CO_2. At 2 days in culture, serum-containing medium was replaced with serum-free medium (DMEM supplemented with ITS-X (GIBCO) containing insulin, transferrin, and sodium selenite (DMEM/ITS)) in the presence or absence of TBT. Cultured neurons were exposed to TBT for 48 hr.

Primary neuron-astrocyte mix culture

Cerebral cortical cells from postnatal day 1 (P1) rats were subjected to primary neuron-astrocyte mix culture. Whole neonatal brains were dissected under sterile condition and cultured cells were prepared as described above (*primary neuron culture*). In this culture, culture medium was DMEM supplemented with 10% FBS (DMEM/10%FBS) and cells were plated at

2,000 cells/mm^2. At 7 days in culture, DMEM/10%FBS was replaced with DMEM/ITS in the presence or absence of TBT, and cultured neurons and astrocytes were exposed to TBT in DMEM/ITS for 48 hr.

Primary astrocyte culture

Cerebral cortical cells were prepared in the same manner as described in *primary neuron-astrocyte mix culture*, and plated onto appropriate culture dishes coated with poly-L-lysine (GIBCO) at 200 cells/mm^2. At 14 days in culture, DMEM/10%FBS was replaced with DMEM/ITS in the presence or absence of TBT, and cultured primary astrocytes were exposed to TBT in DMEM/ITS for 48 hr. It should be noted that astrocytes, unlike neurons, possess proliferation potency *in vitro* and they became dominant during 14 days of culture (see *Results*).

Passaged astrocyte culture

Cerebral cortical astrocytes were obtained by sub-culturing cells in *primary astrocyte cultures*. Cortical astrocytes were allowed to grow to confluence for two weeks *in vitro*. One-half of the volume of the medium was replenished with fresh culture medium twice a week. Astrocytes in confluent cultures were detached from the culture flask by 0.1% trypsin (GIBCO), and sub-cultured in normal plastic culture dishes. This procedure was repeated several times to obtain sufficient number of astrocytes to perform experiments. Before TBT exposure, passaged astrocytes were passaged and plated onto appropriate culture dishes at 200 cells/mm^2 in DMEM/10%FBS. At 5 days after final passage, DMEM/10%FBS was replaced with DMEM/ITS in the presence or absence of TBT, and passaged astrocytes were exposed to TBT in DMEM/ITS for 48 hr.

Assessment of cell viability

Viability of cells cultured in 96-well plates under various conditions was assessed using CellTiter-Blue™ Cell Viability Assay kit (Promega, Fitchburg, WI, USA) which measures mitochondrial metabolic activity of viable cells. CellTiter-Blue™ Reagent was added directly to culture medium in 96-well plates and incubated for 30 min at 37°C. After incubation, fluorescence (560Ex/590Em) was recorded using a Fluoroskan Ascent FL (Thermo Labsystems, Beverly, MA, USA).

Immunocytochemistry

Cells in 16-well chamber slides were fixed with PBS containing 4% paraformaldehyde (Sigma-Aldrich) (PFA) and 8% sucrose (Wako pure chemicals, Osaka, Japan) for 5 min at 4ºC, rinsed in PBS, and post-fixed for 2 min in methanol at -20°C. Fixed cells were incubated with blocking buffer (4% normal goat serum, 2% BSA and 0.2% TritonX-100 in PBS) for 30 min. Subsequently, cells were incubated with blocking buffer containing anti-MAP2 antibody (mouse monoclonal, 1:1000) (Sigma-Aldrich), anti-GFAP (chicken polyclonal, 1:1000) (abcam, Cambridge, UK), or anti-S100β (rabbit monoclonal, 1:1000) (abcam) for overnight at 4°C. They were further incubated with Alexa488-conjugated anti-mouse IgG (1:500) (Invitrogen, Carlsbad, CA, USA), Alexa546-conjugated anti-rabbit IgG (1:500) (Invitrogen), Alexa 546-conjugated anti-chicken IgG (1:500) (Invitrogen) and 0.1 μg/mL Hoechst 33258 (Sigma-Aldrich) for 1 hr. The slides were observed under a fluorescence microscope (Axioplan 2), and the images were recorded with a color CCD camera (ProgRes).

Western blotting

Western blotting was performed as previously described (Oyanagi *et al*., 2015). Briefly, cell homogenate in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris, 2.0% SDS, 10% glycerol, 10% 2-mercaptoethanol, and bromophenol blue) containing protease and phosphatase inhibitors was developed by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred on to polyvinylidene fluoride membrane (Immobilon P; Merk Millipore, Darmstadt, Germany). Protein-blotted membranes were incubated with blocking buffer; 2.5% nonfat dry milk or 2% BSA Tris-buffered saline containing 0.1% Tween20 (TBS-T) and then incubated with one of the following primary antibodies: anti-GFAP antibody (chicken polyclonal, 1:2000) (abcam), or anti-S100β antibody (rabbit monoclonal, 1:2000) (abcam), or anti-β-actin antibody (mouse monoclonal, 1:20000) (Sigma-Aldrich). Further, membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) (Jackson Immunoresearch, West Grove, PA, USA) and protein interested was visualized using Immmobilon Western Chemiluminescent HRP substrate (Millipore) onto Hyperfilm ECL (GE Healthcare, Piscataway, NJ, USA). The density of each band was measured in Image J (NIH), and the results were normalized with β-actin as an internal loading control.

Statistical analyses

Effects of TBT exposure on the cell numbers and protein expression were analyzed by student's t-test and one-way analysis of variance (ANOVA) and following Dunnett's test for multiple comparisons using SigmaPlot 12 (Systat Software, Inc., San Jose, CA, USA), respectively. Cytotoxicity of TBT in each culture condition was

analyzed by ANOVA and following Dunnett's test. Half maximal inhibitory concentrations (IC_{50}) of TBT in each culture condition was calculated after fitting four parameter logistic regression model to cell viability data sets using SigmaPlot 12. In all statistical analyses, α was set at 0.05.

RESULTS

Four types of cell culture were prepared from the rat cerebral cortex to evaluate cell-type-specific and differentiation-status-dependent variations of cytotoxicity of TBT in neurons and astrocytes (see *Materials and Methods* for detailed procedures); primary neuron culture (> 95% neurons), primary neuron-astrocyte mix culture (67% neurons and ≈33% astrocytes), primary (not passaged) astrocyte culture (> 95% astrocytes), and passaged astrocyte (≈100% astrocytes). These cultured neural cells were exposed to various concentrations of TBT in the serum-free medium (DMEM/ITS) for 48 hr.

Effect of TBT on cortical neurons in primary neuron culture

Primary neuron cultures were prepared from E18 rat fetal cerebral cortices, so that > 95% of viable cells cultured were neurons reflecting *in vivo* population of neurons at this developmental stage. Purity of neurons was confirmed by immunocytochemistry for NeuN (Fig. 1A). In primary neuron culture, TBT at 200 nM, but not at 100 nM, decreased the number of nuclei visualized by Hoechst 33258 and NeuN-positive neuronal nuclei (Fig. 1A). TBT at 200 and 400 nM showed statistically significant cytotoxicity in neurons in primary neuron culture (Fig. 1B), in which the four-parameter logistic regression model indicated that IC_{50} of TBT was 198 nM (151 and 382 nM in each of 2 separate experiments).

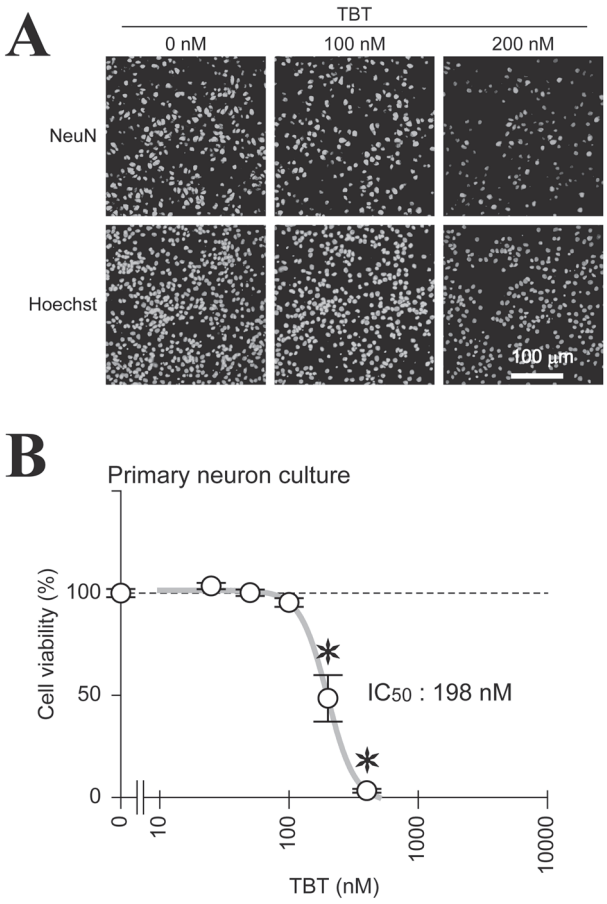

Fig. 1. Effect of TBT on cortical neurons in primary neuron culture. (A) Immunocytochemistry for NeuN (neuron-specific nuclear protein/Fox3) (upper) and visualization of all nuclei with Hoechst 33258 (lower). (B) Cytotoxicity of TBT towards cortical neurons in primary neuron culture evaluated by mitochondrial reducing activity following 48 hr of exposure. Each circle symbol and error bar represent mean and SEM, respectively (n = 8 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control).

Effect of TBT on cortical astrocytes cultured with neurons in primary neuron-astrocyte mix culture

Cerebral cortices of P1 rats yielded viable neurons and astrocytes (two-thirds neurons and the large majority of the remaining one-third astrocytes) in primary neuron-astrocyte mix culture, which roughly reflects the *in vivo* population ratio of neurons and astrocytes at P1. In primary neuron-astrocyte mix culture, TBT at 100 nM diminished MAP-2 positive neurites of neurons (Fig. 2A). TBT at 200 nM decreased the number of MAP-2-positive neurons and TBT at 400 nM induced annihilation of MAP-2-positive neurons (Fig. 2A). TBT at 200 nM induced a slight decrease in the number of total, Hoechst 33258-positive, nuclei and a significant decrease in that of NeuN-positive nuclei (Fig. 2B). Immunocytochemistry revealed that astrocytes in primary neuron-astrocyte mix culture abundantly expressed GFAP and S100β proteins, major astrocyte markers (Fig. 2C). Although TBT at lower doses (< 400 nM)) hardly affected GFAP-positive astrocytes, Western blotting revealed that TBT at 400 nM significantly decreased GFAP expression (Fig. 2D). TBT at 800 nM decreased the number of GFAP-positive astrocytes and induced substantial annihilation of viable cells (data not shown), which was similar in the following cell viability assay. TBT at

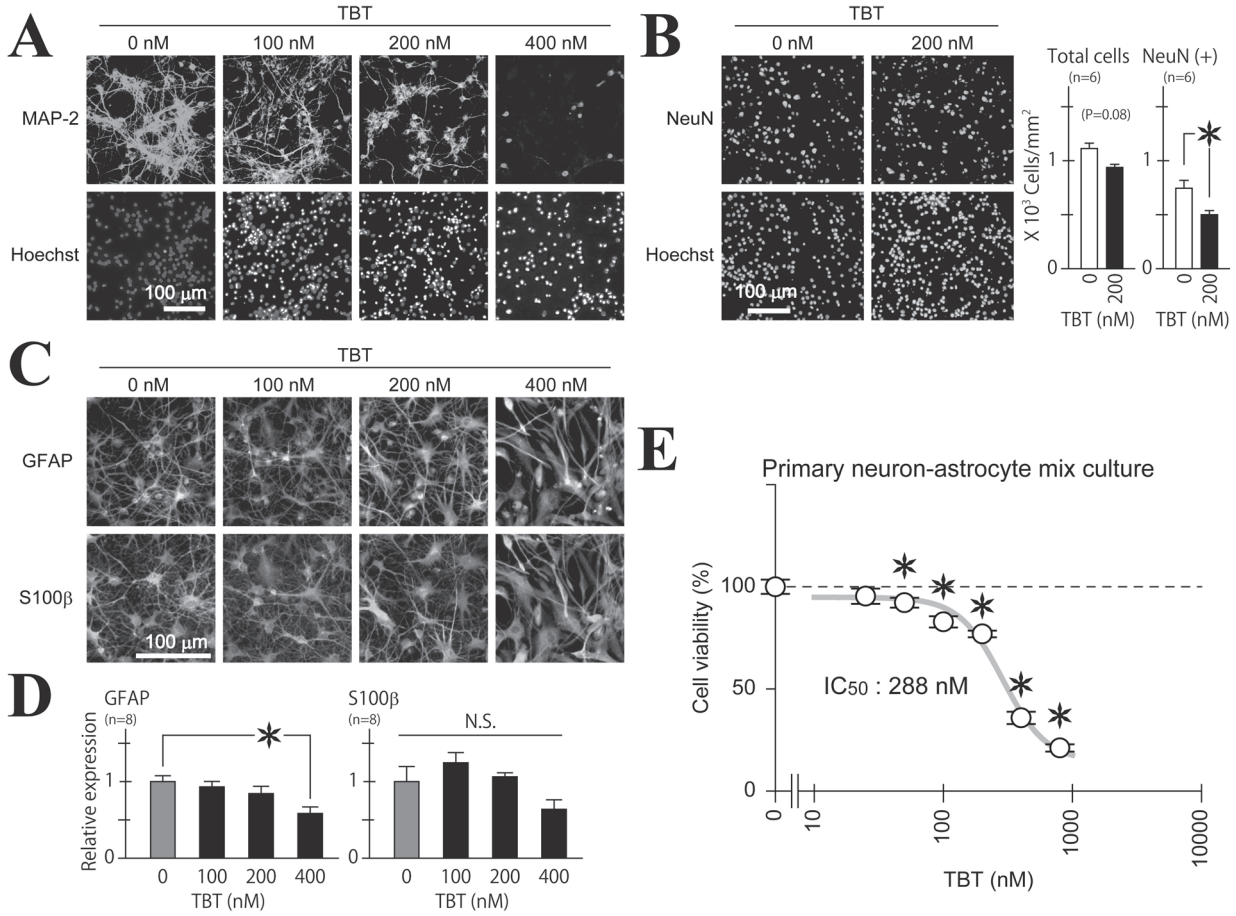

Fig. 2. Effect of TBT on cortical astrocytes cultured with neurons in primary neuron-astrocyte mix culture. (A) Immunocytochemistry for MAP2 (upper) in primary neuron-astrocyte mix culture. Nuclei were stained with Hoechst 33258 (lower). (B) Immunocytochemistry for NeuN (upper) and nuclei visualized with Hoechst 33258 (lower) in cells exposed to TBT at 200 nM and the number of total nuclei and NeuN-positive nuclei. Values were expressed as mean + SEM (n = 6 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control). (C) Immunocytochemistry for GFAP (upper) and S100β (lower) in primary neuron-astrocyte mix culture. (D) Protein expression of GFAP (left) and S100β (right) in cells in primary neuron-astrocyte mix culture. Values were expressed as mean + SEM (n = 8 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control). (E) Cytotoxicity of TBT towards cortical neural cells in primary neuron-astrocyte mix culture evaluated after 48 hr of exposure. Each circle symbol and error bar represent mean and SEM, respectively (n = 12 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control).

50-200 nM induced slight but significant dose-related decrease in cell viability in primary neuron-astrocyte mix culture (Fig. 2E). Higher doses of TBT at 400 and 800 nM led to remarkable and statistically significant reduction in cell viability (Fig. 2E). The IC_{50} of TBT in primary neuron-astrocyte mix culture was 288 nM (291 and 289 nM in each of 2 separate experiments).

Effect of TBT on cortical astrocytes cultured without neurons in primary astrocyte culture

In primary astrocyte culture consisting of cells having proliferated during 14 days, astrocytes were visualized by S100β because immunocytochemical staining of GFAP was weak due to its low expression and, unlike S100β, even imaging astrocyte morphology by GFAP was difficult. Indeed, GFAP and S100β protein expression level in primary astrocyte culture without TBT exposure was 57% and 55%, respectively, of that of each protein

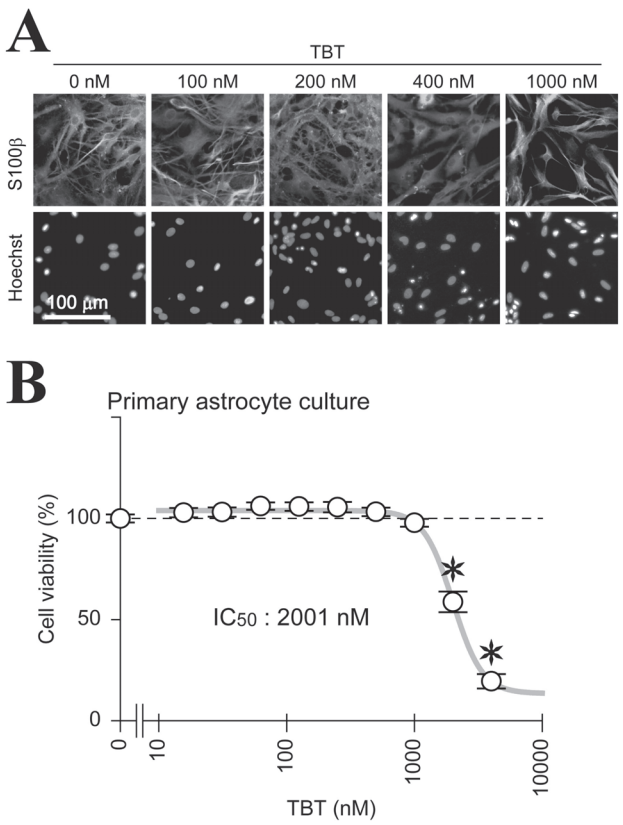

Fig. 3. Effect of TBT on cortical astrocytes cultured without neurons in primary astrocyte culture. (A) Immunocytochemistry for S100β (upper) in primary cultured astrocytes. Nuclei were stained with Hoechst 33258 (lower). (B) Cytotoxicity of TBT towards cortical astrocytes in primary astrocyte culture evaluated after 48 hr of exposure. Each circle symbol and error bar represent mean and SEM, respectively (n = 12 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control).

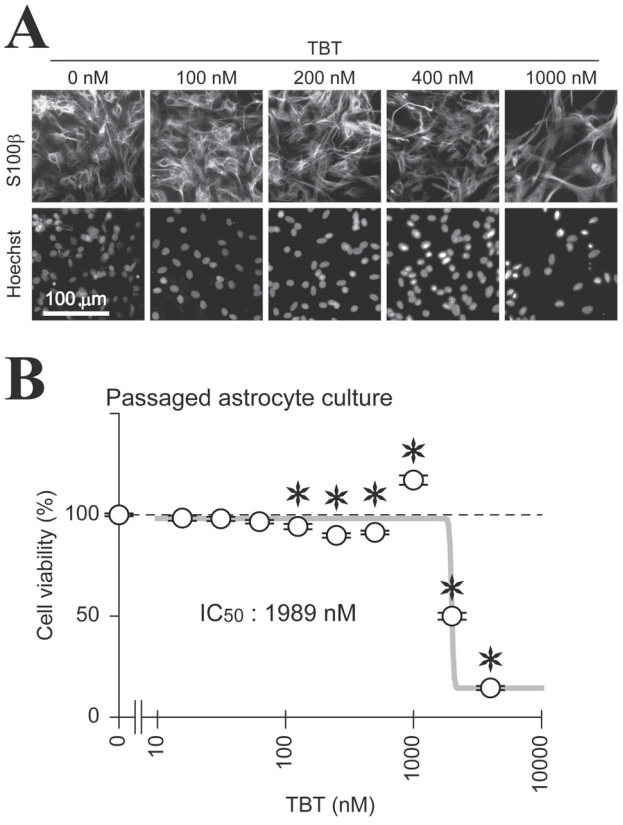

Fig. 4. Effect of TBT on cortical astrocytes in passaged astrocyte culture. (A) Immunocytochemistry for S100β (upper) in passaged astrocytes. Nuclei were stained with Hoechst 33258 (lower). (B) Cytotoxicity of TBT towards mature cortical astrocytes in passaged astrocyte culture after 48 hr of exposure. Each circle symbol and error bar represent mean and SEM, respectively (n = 12 from 2 separate experiments). *; $P < 0.05$ vs 0 nM (vehicle control).

in primary neuron-astrocyte mix culture (Supplementary Fig. 1A). TBT at 400 or 1000 nM seemed to affect the morphology of S100β-positive astrocytes (Fig. 3A), in which astrocytes changed from flat and ramified to spindle shape. TBT at 2000 nM or higher showed statistically significant cytotoxicity in astrocytes in primary astrocyte culture, in which IC_{50} of TBT was 2001 nM (1963 and 2045 nM in each of 2 separate experiments) (Fig. 3B). TBT at 100-400 nM induced a slight, but not significant, dose-dependent decreases in GFAP expression in astrocytes in primary astrocyte culture (Supplementary Fig. 1A).

Effect of TBT on cortical astrocytes in passaged astrocyte culture

Astrocytes in passaged astrocyte culture were also visualized by S100β by the similar reason as in primary astrocyte culture. GFAP and S100β protein expression level in passaged astrocyte culture without TBT exposure was 25% and 55%, respectively, of the level of each protein in primary neuron-astrocyte mix culture (Supplementary Fig. 1B). Interestingly, TBT at 200 nM or higher doses induced morphological changes from amoeboid to asteriated or spindle shape (Fig. 4A). In addition, in passaged astrocyte culture, TBT influenced cell viability in different ways depending on its dose. TBT at 125-500 nM induced

a slight but significant decrease in cell viability, whereas TBT at 1000 nM induced a significant increase (Fig. 4B). At higher doses of 2000 and 4000 nM, TBT induced statistically significant cell loss in passaged astrocytes and IC_{50} of TBT was 1989 nM (1992 and 1987 nM in each of the 2 separate experiments) (Fig. 4B). TBT induced a slight, but not significant, dose-dependent decreases in GFAP and S100β expression in astrocytes in passaged astrocyte culture (Supplementary Fig. 1B).

DISCUSSION

In the present study, we demonstrated cell-type-specific and differentiation-status-dependent variations of cytotoxicity of TBT in four frequently used neural cell culture systems; primary neuron culture, primary neuron-astrocyte mix culture, primary astrocyte culture, and passaged astrocyte culture.

Variations in cytotoxicity of TBT by cell type abundance (comparison of IC_{50})

The concentration of TBT that gives half maximal response (cytotoxicity), IC_{50}, was calculated based on the four-parameter logistic regression model in the present study to evaluate cytotoxic potencies of TBT in four different neural culture systems. TBT exerted the lowest IC_{50} (the highest cytotoxic potency; 198 nM) against neurons in primary neuron culture. In general, neurons are more vulnerable than astrocytes when exposed to toxic substances or undesired conditions such as polybrominated diphenyl ether mixture (Giordano *et al*., 2008), increased intracellular Zn^{2+} (Dineley *et al*., 2000), or brain injury such as ischemia (Thoren *et al*., 2005). In other words, astrocytes are considered more resistant to various stresses as well as exposures to environmental chemicals and neurodegenerative conditions, and that is why astrocytes can support and protect neurons. These concepts hold true in the IC_{50}'s of TBT obtained in the present study. As expected, IC_{50}'s of TBT were high both in primary astrocyte culture (2001 nM) and in passaged astrocyte culture (1989 nM), which suggests that astrocytes are robust against TBT exposure compared to neurons. In primary astrocyte culture and passaged astrocyte culture, cell density at the time of plating was 200 cells/mm^2, while initial cell densities were 3000 and 2000 cells/mm^2 in primary neuron culture and primary neuron-astrocyte mix culture, respectively. As a consequence, astrocytes in primary astrocyte culture and passaged astrocyte culture were proliferative cells which were allowed to proliferate to be almost confluent at the time of TBT exposure. Some trophic factors derived from FBS strongly supported proliferation of astrocytes. Although astrocytes might be intrinsically resistant to exposure to TBT, we considered that astrocytes having high proliferation potency or undergoing cell proliferations many times became robust against TBT exposure. It would be important to assess the relationship between the number of proliferations and the vulnerability to exposure to TBT in proliferating astrocytes in further study.

On the other hand, IC_{50} of TBT in primary neuron-astrocyte mix culture was 288 nM, which was comparable to that in primary neuron culture (198 nM). Interestingly, in spite of comparable IC_{50}'s, the slope of regression curve in primary neuron-astrocyte mix culture was gentler than that in primary neuron culture. In primary neuron-astrocyte mix culture, 67% of total viable cells were neurons and astrocytes comprised almost all of the remaining population. In this mix culture condition, some cells (36%) were alive against TBT at 400 nM, while neurons in primary neuron culture were annihilated by the same dose of TBT, which might be due to the survival of astrocytes that were less vulnerable to exposure to TBT. However, albeit TBT at 100 nM or less showed no neuronal cytotoxicity in primary neuron culture, TBT at these lower doses induced slight but statistically significant decrease in cell viability in primary neuron-astrocyte mix culture. There is a possibility that astrocytes increased neuronal vulnerability to TBT as immunocytochemistry indicated at least in a certain population of neurons like GABAergic inhibitory neurons (Yamada *et al*., 2010). Neuron-astrocyte interaction is an important topic not only in neuroscience but also in toxicology. Especially, recent studies revealed that astrocyte was a major supplier of inflammatory cytokines (IL-6 etc.) (Van Wagoner *et al*., 1999) and chemokines (MCP-1 etc.) (Madrigal *et al*., 2010; Negishi *et al*., 2012) as well as well-known growth factors trophic to neurons such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF). It is important to measure these soluble factors released from astrocytes in to the culture medium, because production of these factors in astrocytes could be affected by exposure to chemicals such as TBT, which might account for increased neuronal vulnerability to TBT in neuron-astrocyte culture. Further experiments with conditioned medium are also interesting in order to discuss neuron-astrocyte interactions.

And furthermore, in primary neuron-astrocyte mix culture, cell viability assays indicated that 80% of total cells were killed by TBT at 800 nM, which would mean that any cells besides neurons, i.e. astrocytes, also died. However, TBT at 800 nM had no or a little cytotoxicity in astrocytes cultured in primary astrocyte culture and pas-

saged astrocytes. These results suggest that, in astrocytes in primary astrocyte culture, a subpopulation having high vulnerability to TBT exists, or that some astrocytes might be made vulnerable to TBT by neurons cultured together via direct and/or indirect interactions.

Differential dose-related cytotoxicity of TBT towards primary cultured astrocytes and passaged astrocytes

Astrocytes were dominant in primary astrocyte culture, majority of which consisted of cells that had grown and proliferated in 14 days since being seeded, and they were much more resistant to TBT (IC_{50} was 2001 nM) than neurons in primary neuron culture (IC_{50} was 198 nM) or cells in primary neuron-astrocyte mix culture (IC_{50} was 288 nM). In addition, passaged astrocytes also showed potent resistance to TBT (IC_{50} was 1989 nM), which was comparable to astrocytes in primary astrocyte culture. However, the characteristics of dose-related TBT cytotoxicity were different between primary astrocytes and passaged astrocytes. Although TBT is a widely accepted toxic substance, TBT at 1000 nM increased cell viability of astrocytes in passaged astrocyte culture unlike in primary astrocyte culture. Thus, the calculated regression curve for passaged astrocytes showed a slope steeper than that for astrocytes grown in primary astrocyte culture. These differences might be due to the homogeneity, i.e. clonality, of cultured astrocytes. Passaged astrocytes had proliferated and grown more than astrocytes in primary cultured astrocytes, where selection of highly proliferating astrocytes would occur consequently. It is nothing else but an increase in homogeneity of astrocytes based on their proliferative activity, which might lead to steep dose-dependent, nearly all-or-none, TBT cytotoxicity. By the similar reason, there is a possibility that, against possible stress of TBT at 1000 nM, these passaged astrocytes could accelerate their proliferation or increase their cellular metabolic activities which convert resazurin into resorufin in cell viability assays all at once. It is important to elucidate the molecular mechanism of this increased cell viability by TBT at 1000 nM in further studies. Consequently, the logistic regression curve in passaged astrocyte culture showed low goodness of fit when compared to those in other three cultures because of decrease in cell viability by TBT at 250 nM and increase by TBT at 1000 nM, which might cause some uncertainty in calculation of IC_{50}. However, the calculated IC_{50} of 1989 nM in passaged astrocyte culture was thought to be relevant, because the numerical mean value of cell viability at 2000 nM TBT in passaged astrocyte culture was 49.9%. More detailed dose-relationship should be examined in further study.

TBT is known as an environmental endocrine disrupting chemical. In many marine species, TBT induced male sexual characteristics in females by decreasing their aromatase activity (McAllister and Kime, 2003). In mammals including humans, TBT affected not only immune (Markovič *et al*., 2015), metabolic (Janesick and Blumberg, 2012; Nakanishi, 2008; Chamorro-Garcia *et al*., 2013), and reproductive systems (Mitra *et al*., 2013; Graceli *et al*., 2013) but also the CNS (Asakawa *et al*., 2010; Mitra *et al*., 2014; Hasegawa *et al*., 2013; Tsunoda *et al*., 2006; Konno *et al*., 2005). Although comprehensive molecular mechanisms of TBT toxicity are largely unknown, these recent studies and others have proposed various molecular targets of TBT. Among them, peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor complex (Hiromori *et al*., 2014), AMP-activated protein kinase (Yamada *et al*., 2013) are especially important when considering adverse effects of TBT on the CNS because neurons and astrocytes in the CNS abundantly express and effectively utilize these proteins (Gatson *et al*., 2011; Taib *et al*., 2013; Aleshin *et al*., 2009; Ronnett *et al*., 2009). On the other hand, TBT was also known as a potent inhibitor of F-ATP synthase (von Ballmoos *et al*., 2004; Nesci *et al*., 2014), which would be also important because inhibition of cellular ATP synthesis results in cell death immediately. Although we focused only on TBT in the present study, previous studies revealed that trimethyltin (TMT) and triethyltin (TET) had a potent adverse effect specifically on microglia and astrocytes *in vitro* in rats (Rohl *et al*., 2009) and TMT on granule neurons in murine hippocampus (Shintani *et al*., 2007). Thus, it is of great interest to examine the adverse effects of TMT and TET on neurons and astrocytes in cultures provided in the present study. Triphenyltin (TPT) has also been used as algicides and molluscicides in antifouling product together with TBT. Taken together, further study considering structure-activity relationship among TMT, TET, TBT, and TPT would give us a deeper understanding of toxicity of organotins in the CNS.

Although, in the present study, we focused mainly on the cytotoxicity of TBT, evaluations based on the specific mechanism of TBT toxicity should be provided in future studies. In addition, biological or toxicological mechanism of neuron-astrocyte interactions implied in the present study should be elucidated. This is a pioneering study demonstrating variable cytotoxicity of TBT in neural cells depending on the culture condition.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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