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# Cytotoxicity of tributyltin in rat hippocampal slice cultures

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**Abstract**

The neurotoxic effects of tributyltin (TBT), an endocrine-disrupting chemical, were evaluated in organotypic slice cultures of immature rat hippocampus. Confocal microscopy study with propidium iodide showed that TBT induced severe neuronal death in a concentration- and time-dependent manner with CA3 > CA1 > dentate gyrus ranking of vulnerability of the hippocampal subfields. Dead or damaged neurons exhibited chromatin condensation, which is one of the morphological characteristics of apoptosis, as revealed by acridine orange staining. TBT neurotoxicity was alleviated by application of free radical scavengers or antioxidants, such as catalase, superoxide dismutase, Trolox and α-tocopherol but not by ascorbic acid or *N*-acetyl-L-cysteine, which suggests an involvement of free radicals, particularly reactive oxygen species. Neurons displayed a long-lasting increase in intracellular $Ca^{2+}$ concentrations after TBT treatment. Although neither *N*-methyl-D-aspartate (NMDA) receptor inhibitors nor voltage-sensitive $Ca^{2+}$ channel blockers protected hippocampal neurons against TBT neurotoxicity, non-NMDA receptor antagonist completely prevented TBT-induced neurodegeneration. These data suggest that TBT provokes apoptosis-like neuronal cell death, which might be mediated by intracellular $Ca^{2+}$ elevation and free radical generation via non-NMDA receptor activation. 

*Keywords:* Organotin; Tributyltin; Neurotoxicity; Hippocampus; Neurodegeneration

## 1. Introduction

Although tributyltin (TBT), one of the organotin compounds, has been widely utilized in the production of biocides and in the stabilization of polyvinyl chloride for a long period of time (Piver, 1973), it has recently received much attention as an endocrine-disrupting chemical because of its environmental and health hazards. The compound develops a concentration- and time-dependent 'imposex', which consists of a superimposition of male characteristics onto a reproductive anatomy in female animals of the dioecious snail such as *Nassarius obsoletus Say* (Smith, 1981) and *Neogastropod molluscs* (Horiguchi et al., 1998). This anatomical aberration is likely a consequence of the inhibition by TBT of aromatase cytochrome P450, which catalyzes the aromatization of androgens to estrogens (Bettin et al., 1996; Sumpter, 1998).

Although there are several reports indicating that intoxication of humans or experimental animals with organotin compounds causes epilepsy and amnesia (Kreyberg et al., 1992; Feldman et al., 1993; Tsunashima et al., 1998), the toxicity for mammals is not completely elucidated. Very recently, we showed that TBT possessed diverse pharmacological properties in mammalian tissues (Mizuhashi et al., 2000). Therefore, it is plausible that TBT expresses an unidentified effect on humans because TBT might accumulate in the body, and even be passed on to descendants through food intake such as fish and shellfish inhabiting TBT-polluted environments (Ueno et al., 1999). Experimentally, TBT has also been used extensively as a research tool because of its potent pro-apoptotic action on various types of cells, including thymocytes (Aw et al., 1990; Raffray et al., 1993; Zucker et al., 1994), hepatocytes (Reader et al., 1999), neurons (Thompson et al., 1996) and pheochromocytoma PC12 (Kelve et al., 1994; Lauc et al., 1994; Viviani et al., 1995). However, few previous studies have addressed the effect of TBT on the nervous system whereas organotins might induce

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epilepsy and amnesia (Feldman et al., 1993; Lauc et al., 1994; Tsunashima et al., 1998). Because the hippocampal formation is associated with epilepsy and cognitive ability (Reid and Stewart, 1997; Roman et al., 1999), the present study investigated the effects of TBT on hippocampal neurons using organotypic slice cultures. Here we found that TBT caused neuronal cell death in a concentration- and time-dependent manner. Furthermore, we evaluated the mechanisms of the TBT neurotoxicity.

## 2. Materials and methods

### 2.1. Organotypic slice cultures

The hippocampi were dissected from postnatal 10-day-old Wistar rats (SLC, Shizuoka, Japan) and cut into 300-μm-thick slices using a microslicer (DTK-1500, Dosaka EM, Osaka, Japan) under sterile conditions. The slices were placed on polytetrafluoroethylene membranes (Millicell™-CM, Millipore, Bedford, MA) which were inserted into six-well plates filled with culture medium consisting of 50% minimum essential medium (Life Technologies, Gaithersburg, MD), 25% Hanks' balanced salt solution and 25% horse serum (Cell Culture Lab, Cleveland, OH). The cultures were kept at 37°C in a humidified and 5%-$CO_2$ atmosphere, and the culture medium was changed every 3.5 days. The cultures were used in experiments at 10–12 days in vitro (DiV).

### 2.2. Exposure to TBT

Cultures were exposed for 2–36 h to fresh TBT (tributyltin chloride, Wako Pure Chemical Industries, Saitama, Japan) dissolved in $< 0.02\%$ dimethyl sulfoxide (DMSO) (Wako) in concentrations ranging from 0.1 to 10 μM.

### 2.3. DiI staining

The slices were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde for 30 min, and then a fluorescent membrane dye DiI crystal was carefully placed on the stratum pyramidale. Following incubation in the fixative at room temperature for 2 days, DiI-labeled cells were observed using a confocal microscope. Under these conditions, the cells were labeled with DiI in a random fashion and thereby were able to be observed individually.

### 2.4. PI uptake

Propidium iodide (PI) is a polar compound which only enters cells with damaged membranes and becomes brightly red fluorescent after binding to nucleic acids (Macklis and Madison, 1990; Vitale et al., 1993; Ikegaya, 1999). Twenty-four hours after 5 μg/ml PI (Molecular Probes, OR) was added to the culture medium, the cultures were photographed and PI fluorescence imaging was performed with a confocal microscope. PI uptake as neurodegeneration index was defined as the ratio of PI fluorescence intensity after TBT treatment to putative maximal value obtained after low-temperature exposure (4°C for 48 h).

### 2.5. Acridine orange labeling

Acridine orange is a fluorescent nuclear marker which allows detection of the nuclei of individual cells even in 300-μm-thick slices. Twenty-four hours after 11-DiV cultures were transferred to the medium containing TBT (0 or 3 μM), the slices were fixed with 0.1 M phosphate buffer containing 8% paraformaldehyde at 4°C for 30 min. After being washed with phosphate-buffered saline at room temperature twice for 15 min, the cultures were exposed to 1.0 μg/ml acridine orange (Sigma, St Louis, MO) for 10 min in order to perform nuclear fluorescent staining. The slices were bathed in distilled water twice for 15 min, and then fluorescence imaging was conducted with a confocal microscope.

### 2.6. Calcium imaging

The 11-DiV cultures were treated with Gaya's balanced salt solution (GYBSS) containing 30 μg/ml Fluo-3 AM (Wako) for 1 h. After slices were washed with GYBSS for 10 min, serial fluorescence images of Fluo-3 were obtained with a real-time confocal microscope system, and fluorescent intensity in the cell body of each neuron was measured as an indicator of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Time course of the fluorescent intensity was expressed as a percentage of the baseline value immediately before TBT treatment.

### 2.7. Confocal microscopy

Confocal imaging was carried out with a Micro Radiance (Biorad, Herculeus, CA) laser scanning confocal system equipped with an ECLIPSE TE300 (Nikon, Tokyo, Japan) inverted microscope, an argon ion laser and a host computer system. All imaging and processing operations were performed with Laser Sharp Acquisition (Biorad) and Laser Sharp Processing (Biorad), respectively. For the measurements of PI or DiI fluorescence, the cultures were illuminated with an excitation wavelength of 514 nm, and the fluorescence images were obtained through a 570-nm band-pass filter. For the measurements of acridine orange or Fluo3 fluorescence, the cultures were illuminated with the excitation wavelengths of 488 nm, and the fluores-

cence images were obtained through a 530-nm band-pass filter. The intensity of fluorescence was assessed as an averaged value of pixel intensity (0–255) within the intended area of hippocampal slices.

### 2.8. Drugs

The following drugs were utilized as pharmacological tools in order to clarify the mechanism of TBT neurotoxicity. Catalase 1000 U/ml (Sigma), superoxide dismutase 200 U/ml (SOD; Wako), Trolox 500 μM (Aldrich, Milwaukee, WI), DL-α-tocopherol 500 μM (Eisai, Tokyo, Japan), ascorbic acid 3 mM (Wako) and *N*-acetyl-L-cysteine 250 μM (NAC; Sigma) were used as free radical scavengers or antioxidants. Nifedipine 100 μM (Wako) and nicardipine 100 μM (Wako) were used as a voltage-sensitive L-type $Ca^{2+}$ channel inhibitor. MK-801 10 μM (RBI, Natick, MA), 6-cyano-7-nitroquinoxaline-2,3-dione 10 μM (CNQX, RBI), 2-amino-5-phosphonopentanoic acid 100 μM (AP-5, Sigma) and kynurenic acid 3 mM (Sigma) were used as *N*-methyl-D-aspartate (NMDA) or non-NMDA glutamate receptor antagonists. The above concentrations of each drug were defined after referring to previously published papers (Muller et al., 1993; Pozzo Miller et al., 1994; Takahashi et al., 1995; Maurer and Wray, 1997a,b; Rivera et al., 1998).

### 2.9. Statistics

All data are expressed as the means ± S.E.M. Statistical significance was assessed with one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple range test.

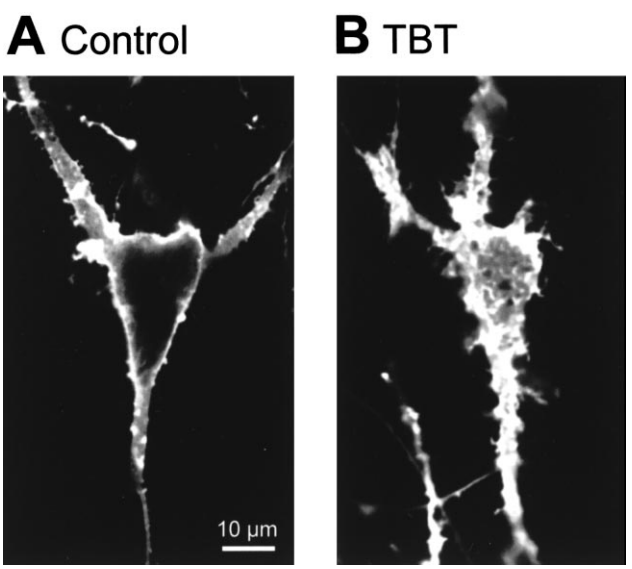


Fig. 1. DiI-labeled neurons cultured in the absence (A) and presence (B) of 5 μM TBT for 24 h. The CA1 pyramidal cells were visualized with DiI and observed with confocal microscopy.

## 3. Results

### 3.1. Neurotoxicity of TBT

The hippocampal slice culture treated with 5 μM TBT for 24 h was labeled with DiI. Confocal microscopy observation revealed that the neurons died with severe damage of the cytomembrane (Fig. 1). Nissl staining also revealed massive loss of the pyramidal cells and the dentate granule cells in the slice treated with TBT. However, because these histochemical methods were inadequate for an accurate quantitation of cell damage, the cell death was assessed by PI fluorescence in the following experiments.

The PI uptake of slices cultivated in vehicle solution containing 0.02% DMSO was very low (Fig. 2A). This result shows that a 24-h exposure to vehicle caused no neurotoxicity in control cultures. However, the slices exposed to 5 μM TBT for 24 h displayed a significant PI uptake (Fig. 2A), which indicates that chronic TBT treatment resulted in severe cell damage in cultures. Therefore, the cell death was evaluated 0, 3, 6, 12, 24 and 36 h after the treatment with 5 μM TBT (Fig. 2B). Cell death appeared 12 h after TBT exposure and then increased gradually. In this experiment, we determined whether the vulnerability to TBT was uniform among the hippocampal subregions, and found that TBT exerted a regionally different neurotoxicity. The order of hippocampal vulnerability was the CA3 region, the CA1 region and the dentate gyrus (DG) ($F(2,126) = 8.050$, $P < 0.001$; two-way ANOVA, Fig. 2B). We next examined the concentration dependency of TBT-induced neurodegeneration. The PI uptake increased with increasing concentration of TBT (0.3, 1, 3, 5, 7 and 10 μM) (Fig. 2C). In the cultures exposed to 0.3 or 1.0 μM TBT for 24 h, PI uptake was faint in the CA1 region, the CA3 region and the DG. But the signal was more pronounced in the cultures exposed to 3.0 μM TBT, and the cultures exposed to higher concentrations of TBT showed significant PI uptake in all hippocampal subregions. A regionally different vulnerability was again noticed with the same order of the hippocampal subregions ($F(2,231) = 7.230$, $P < 0.001$). Taken together, TBT induced a time- and concentration-dependent neuronal death with CA3 > CA1 > DG ranking of vulnerability of the hippocampal subfields.

### 3.2. Acridine orange staining

The dying cells following TBT exposure were stained with acridine orange. Twenty-four hours after TBT treatment, about 40% of the CA3 pyramidal cells exhibited condensed chromatin in the nuclei (Fig. 3). The same morphological appearance was also found in the pyramidal cells of the CA1 region and the granule cells of the DG (data not shown). Since the chromatin

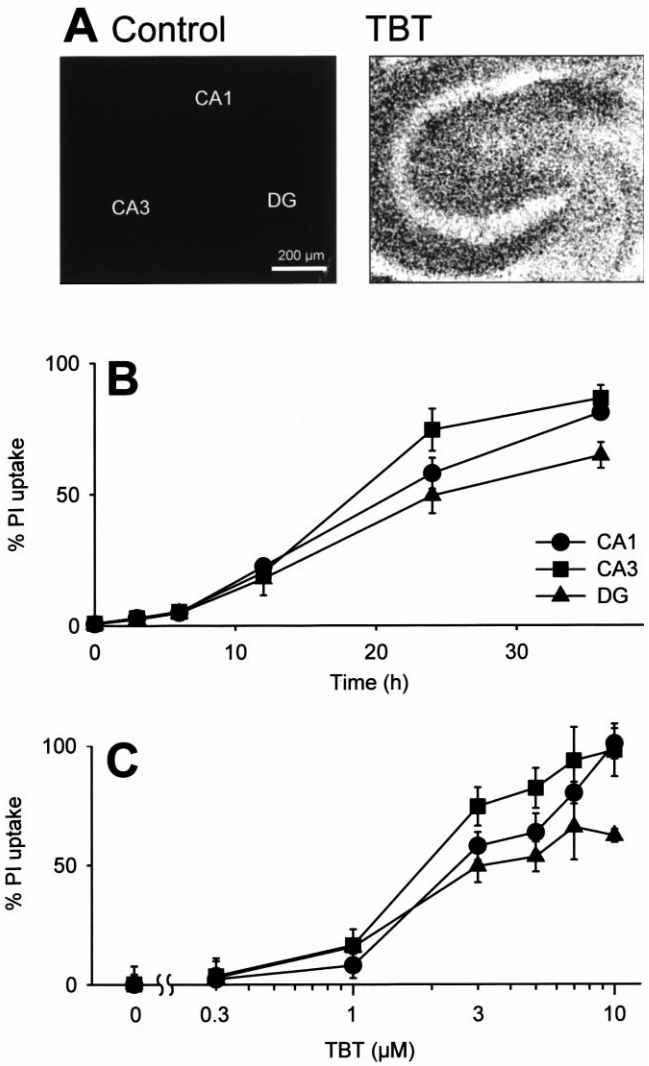


Fig. 2. TBT-induced cell death assessed by PI uptake. (A) Confocal microscopy images of PI fluorescence represent the slices that received the 24-h exposure to 5 μM TBT or its vehicle (Control). Time course (B) and concentration dependency (C) of PI uptake of hippocampal subregions are summarized, i.e. the CA1 area (●), the CA3 area (■) and DG (▼). PI uptake is expressed as a percentage of total uptake after the slice was kept at low temperature. The data are expressed as the means ± S.E.M. of 12 cases.

condensation is a typical characteristic in apoptosis (Walker et al., 1988; Elstein and Zucker, 1994; Elstein et al., 1995), we investigated the effect of cycloheximide and actinomycin D, widely used as apoptosis inhibitors (Walker et al., 1988; Maurer and Wray, 1997a,b; Ben-Ari and Khrestchatisky, 1998), on TBT neurotoxicity in order to determine whether TBT-induced cell death was apoptotic. However, they alone caused severe neurodegeneration in our hippocampal slice cultures, even at low concentrations (3.5 μM cycloheximide and 4 μM actinomycin D), and so we failed to verify the possibility.

### 3.3. Effects of antioxidants

The process of cell injury or cell death including apoptosis often involves free radical generation (Fawthrop et al., 1991; Dubinsky et al., 1995; Chakraborti et al., 1999). Therefore, we first examined the effects of scavenger proteins (catalase and SOD) on TBT toxicity. When they were applied during the 24-h exposure to 5 μM TBT, 1000 U/ml catalase almost completely prevented TBT-induced cell death, 200 U/ml

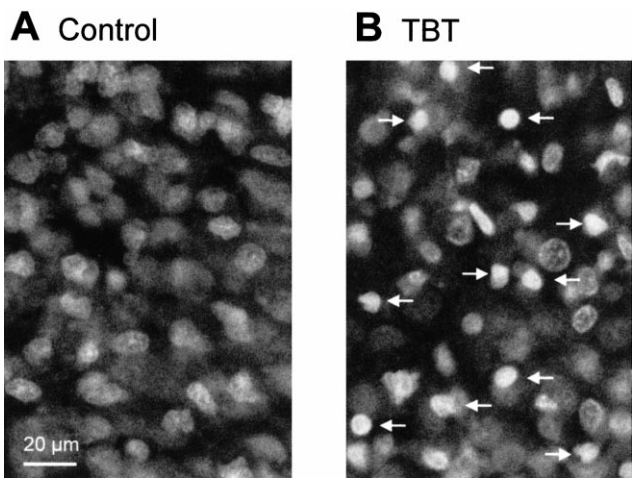


Fig. 3. TBT-induced chromatin condensation. The cell nuclei in cultures exposed to vehicle (A) or 3 μM TBT (B) for 24 h were stained with acridine orange and observed with confocal microscopy. Arrows in panel B indicate condensed chromatin.

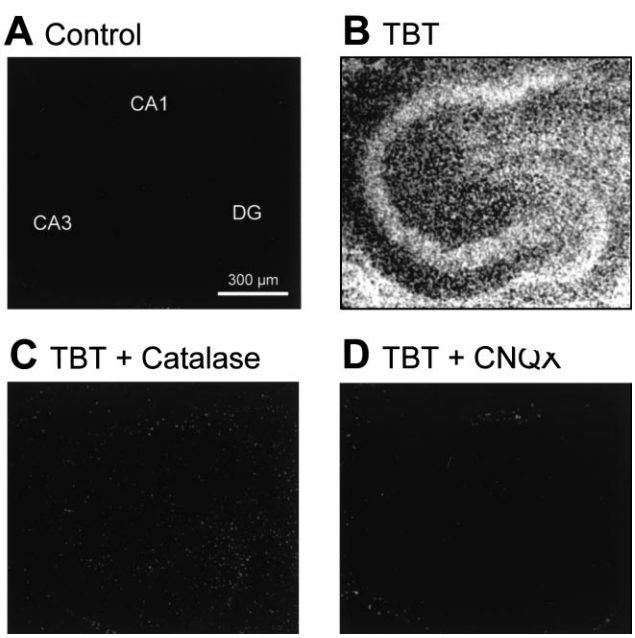


Fig. 4. TBT-induced neuronal cell loss was prevented by catalase or CNQX. During 24-h exposure to vehicle (A) or 5 μM TBT (B), slices were cotreated with 1000 U/ml catalase (C) or 10 μM CNQX (D), and subsequently TBT-induced cell death was assessed by PI uptake with a confocal microscope.

Table 1
Effects of various antioxidants on TBT-induced cell death in hippocampal slice cultures[a]

| Drug | Concentration | % Inhibition of TBT toxicity | | |
|---|---|---|---|---|
| | | CA1 | CA3 | DG |
| Catalase | 1000 U/ml | 88.93 ± 3.72* | 91.37 ± 10.82** | 89.80 ± 4.93* |
| SOD | 200 U/ml | 30.99 ± 3.95 | 2.64 ± 3.20 | 71.95 ± 4.57* |
| Trolox | 500 μM | 58.74 ± 11.70** | 70.47 ± 11.74** | 31.37 ± 1.98 |
| Tocopherol | 500 μM | 32.68 ± 19.54 | 35.15 ± 16.58 | 28.78 ± 7.88 |
| Ascorbic acid | 3 mM | 0.00 ± 0.00 | 0.00 ± 0.00 | 7.69 ± 2.80 |
| NAC | 250 μM | 3.40 ± 6.09 | 0.00 ± 0.00 | 0.00 ± 0.00 |

[a] PI uptake was measured after 24-h exposure to 5 μM TBT in normal medium or in the presence of catalase, SOD, Trolox, tocopherol or NAC. The rate of inhibition of TBT toxicity was calculated by {1 − (the value in drug-treated slice − the value in naive slice)/(the value in the slice treated with TBT alone − the value in naive slice)} × 100. The data represent the means ± S.E.M. of 8–12 slices.

* $P<0.05$, Tukey's test following one-way ANOVA as compared to the culture exposed to TBT alone.

** $P<0.01$, Tukey's test following one-way ANOVA as compared to the culture exposed to TBT alone.

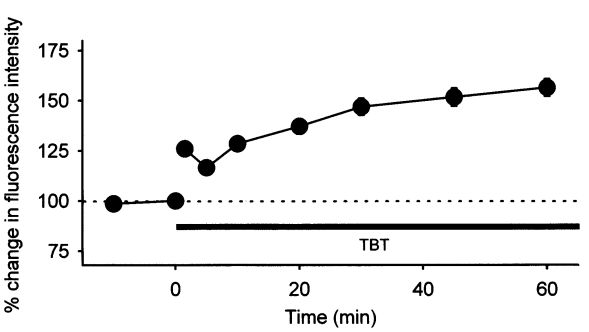


Fig. 5. TBT increased $[Ca^{2+}]_i$ of the pyramidal cells. TBT (3 μM) was applied to cultures pre-loaded with Fluo-3, and changes in fluorescence intensity were estimated with a real-time confocal microscopy system. The ordinate was expressed as a percentage of the baseline value immediately before the treatment with TBT. Vertical bars on data points are S.E.M. of 30 cases; when not indicated, S.E.M. fell within the data symbols.

and SOD also did slightly but the effect was rather feeble (Fig. 4, Table 1). The result suggests that the toxicity is mediated by reactive oxygen species, which might contain $H_2O_2$ more predominantly than $O_2^-$. Furthermore, the action of lipid-soluble antioxidant (Trolox or DL-α-tocopherol) or water-soluble antioxidant (ascorbic acid or NAC) was also studied (Table 1). Trolox (500 μM) significantly attenuated TBT-induced cell death. α-Tocopherol (500 μM) also lowered TBT neurotoxicity but its effect was not significant. The higher concentration of α-tocopherol was also tested but a similar result was obtained (data not shown). Neither 3 mM ascorbic acid nor 250 μM NAC reduced TBT-induced cell death.

### 3.4. $[Ca^{2+}]_i$ measurement

Since certain types of cell death including apoptosis were often preceded or triggered by aberrant $[Ca^{2+}]_i$ elevation (Fawthrop et al., 1991; Chakraborti et al., 1999), we monitored the changes in $[Ca^{2+}]_i$ after TBT exposure using Fluo-3, a $Ca^{2+}$ indicator. When cultures were treated with TBT, the pyramidal cells displayed a $[Ca^{2+}]_i$ increase which consisted of a rapid and transient rise within a few minutes and subsequently a gradual and long-lasting elevation over 1 h (Fig. 5).

### 3.5. Effects of glutamate antagonists

Because TBT evoked the dynamic $[Ca^{2+}]_i$ alteration, we explored the possible involvement of $Ca^{2+}$-permeable channels in TBT neurotoxicity. First, the effect of an L-type $Ca^{2+}$ channel blocker nifedipine was investigated. But 100 μM nifedipine did not rescue TBT-induced cell death (Table 2). Another type of L-type $Ca^{2+}$ channel blocker, 100 μM nicardipine was also ineffective (data not shown). Next, because NMDA receptor activation is well known to increase $[Ca^{2+}]_i$ and thereby to produce hippocampal neuron loss (Michaels and Rothman, 1990; Churn et al., 1995), the effect of NMDA receptor antagonist MK-801 or AP-5

Table 2
Effects of various channel or receptor antagonists on TBT-induced cell death in hippocampal slice cultures[a]

| Drug | Concentration | % Inhibition of TBT toxicity | | |
|---|---|---|---|---|
| | | CA1 | CA3 | DG |
| Nifedipine | 100 μM | 0.00 ± 0.00 | 6.64 ± 8.38 | 0.00 ± 0.00 |
| MK-801 | 10 μM | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| AP-5 | 100 μM | 0.00 ± 0.00 | 0.00 ± 0.00 | 14.36 ± 2.62 |
| CNQX | 10 μM | 75.31 ± 34.16 | 87.31 ± 10.30** | 97.13 ± 5.99* |
| Kynurenic acid | 3 mM | 2.01 ± 8.47 | 36.28 ± 5.50 | 52.47 ± 6.55 |

[a] PI uptake was measured after 24-h exposure to 5 μM TBT in normal medium or in the presence of nicardipine, MK801, AP-5, CNQX or kynurenic acid. The data, defined as described in Table 1, represent the means ± S.E.M. of 8–12 slices.

* $P<0.05$, Tukey's test following one-way ANOVA as compared to the culture exposed to TBT alone.

** $P<0.01$, Tukey's test following one-way ANOVA as compared to the culture exposed to TBT alone.

was examined (Table 2). However, neither 10 μM MK-801 nor 100 μM AP-5 restored TBT-induced cell death. Then we finally checked the effect of CNQX, a competitive antagonist of non-NMDA receptor, and found that 10 μM CNQX dramatically protected hippocampal neurons against TBT neurotoxicity (Fig. 4, Table 2). A non-selective ionotropic glutamate receptor antagonist kynurenic acid 3 mM also prevented TBT-induced cell death but the effect was not significant (Table 2).

## 4. Discussion

Using organotypic cultures of hippocampal slices, we have demonstrated that TBT, an endocrine-disrupting chemical, causes neuronal death with regionally different vulnerability of the hippocampal subfields (CA3 > CA1 > DG) in a concentration- and time-dependent manner. The toxicity is possibly mediated by reactive oxygen species generation and/or by non-NMDA receptor activation. A previous study reported that trimethyltin, an organotin compound with a similar chemical structure, also seriously damaged organotypic rat hippocampal slice cultures (Noraberg et al., 1998). However, the neurotoxicity of trimethyltin appears at concentrations of >40 μM. By contrast, TBT can produce massive cell loss even at 3 μM. Therefore, TBT toxicity is about 10 times more potent. This may be the reason why TBT is apt to become an environmental and health hazard.

The dead or damaged cells after TBT exposure exhibited condensed chromatin in the nuclei while their cytoplasm was considerably preserved. Because this is one of the typical characteristics of apoptosis (Walker et al., 1988; Elstein and Zucker, 1994; Elstein et al., 1995), TBT-induced cell death might be due to an apoptosis-like process. The slow time course of TBT neurotoxicity (>12 h) also suggests apoptotic cell death. However, further experiments would be required to determine whether TBT-induced neuronal cell death is apoptosis.

The result that application of Trolox or α-tocopherol blocked the toxic action of TBT suggests an involvement of free radicals in the toxicity. In particular, reactive oxygen species including $H_2O_2$ and $O_2^-$ presumably play a pivotal role because TBT toxicity was abolished by catalase as well as SOD. Therefore, TBT may produce reactive oxygen species within or around cells and consequently cause the neurodegeneration. However, another possibility can also be raised. Rivera et al. (1992) reported that TBT penetrates the plasma membrane only in the presence of endogenous $O_2^-$ and then induces morphological changes in erythrocytes and hemolysis. Therefore, TBT neurotoxicity may require endogenous $H_2O_2$ and $O_2^-$ rather than TBT itself producing harmful reactive oxygen species. Additionally, we cannot rule out the possible involvement of other types of radicals. Indeed Kergosien and Rice (1998) reported that TBT enhances nitric oxide production in macrophages. Therefore, various radicals including nitric oxide may be, in part, involved in TBT toxicity.

Although Chow et al. (1992) indicated that TBT induced a subsequent $[Ca^{2+}]_i$ increase in thymocytes, we have shown for the first time that neuronal cells displayed a $[Ca^{2+}]_i$ rise in response to TBT. In contrast to the case of thymocytes, the $[Ca^{2+}]_i$ elevation in neurons displayed a biphasic fashion; a rapid and transient rise of $[Ca^{2+}]_i$ was followed by a slow and consecutive increase in $[Ca^{2+}]_i$. Although the mechanism remains to be clarified, the $[Ca^{2+}]_i$ elevation in thymocytes is assumed to be essential for TBT-induced apoptosis (Chow et al., 1992). Therefore, the present study investigated the contribution of $Ca^{2+}$-permeable channels to TBT-induced neurodegeneration. However, neither L-type $Ca^{2+}$ channel inhibitors nor NMDA receptor antagonists protected hippocampal neurons against TBT neurotoxicity. Although the L-type channel is the voltage-sensitive $Ca^{2+}$ channel that is most highly contained in the CA1 pyramidal cell layer, other types of $Ca^{2+}$ channel also exist (Ishibashi et al., 1995). Therefore, we must further examine the effect of other types of channel blockers to investigate the involvement of $Ca^{2+}$ entries in TBT-induced $[Ca^{2+}]_i$ elevation. However, Chow et al. (1992) suggest that endoplasmic reticular $Ca^{2+}$ mobilization might trigger a TBT-evoked $[Ca^{2+}]_i$ rise. It suggests that TBT neurotoxicity may also be provoked by $Ca^{2+}$ release from the intracellular store. Additionally, because biochemical studies indicate that submicromolar TBT decreases the enzyme activities of $Ca^{2+}$-ATPase through the inhibition of calmodulin (Yallapragada et al., 1990, 1991), it is also possible that the $[Ca^{2+}]_i$ increase, particularly the late phase, is accomplished by TBT-induced inhibition of $Ca^{2+}$-ATPase-dependent $Ca^{2+}$ efflux.

The most surprising result obtained in this study is that non-NMDA receptor antagonist showed a protective action against TBT neurotoxicity, but NMDA receptor antagonist did not. This is analogous to brain ischemia, in which administration of non-NMDA receptor antagonist protects the hippocampal neurons against delayed cell death (Sheardown et al., 1990; Siesjo et al., 1991; Sheardown et al., 1993). Furthermore, the ischemic neuron loss is apoptotic and accompanied by free radical generation and aberrant $[Ca^{2+}]_i$ elevation, which is found in the hippocampal pyramidal cells more predominantly than the dentate granule cells (Siesjo et al., 1991; Peruche and Krieglstein, 1993). These appear to be similar to TBT neurotoxicity found in the present study. Although the mechanism underlying TBT-induced cell death is obscure, this analogy may give us diverse strategies to elucidate it. In particu-

lar, the effectiveness of non-NMDA receptor antagonist urges us to explore the action of TBT on glutamate receptor and neuronal excitability. Accordingly, electrophysiological and further pharmacological approaches are now underway in our laboratory.

Our present study has shown that TBT displayed neurotoxicity at relatively low concentrations in hippocampal slice cultures. Because Horiguchi et al. (1994) reported that TBT concentrations in rock shells living in polluted sea areas in Japan reach 1–5 μM, it is possible that TBT is accumulated in mammals as a result of ecological magnification. Indeed, Kannan and Falandysz (1997) indicated that a significant concentration ($\sim$0.4 μM) of butyltin was detected in human liver. The present study may hence give a warning about unconfirmed toxicity of this environmental pollutant for mammals. Indeed, we found that submicromolar TBT augmented the neurotoxicity of amyloid β in vitro and also that it decreased spine density in proximal apical dendrites of the CA1 pyramidal cells (unpublished data). Furthermore, an in vivo study using neonatal rats indicates that TBT decreases myelin basic protein and synaptic vesicle-associated protein p38 at doses that do not induce hippocampal neuron loss (O'Callaghan and Miller, 1988). These suggest that TBT influences the functions of the central nervous system as well as neuron survival. Thus, the organotypic slice culture utilized in the present study may provide a useful system for studying TBT toxicity on the mammalian central nervous system.

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