Note

Prenatal Exposure to Tributyltin Decreases GluR2 Expression in the Mouse Brain

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Tributyltin (TBT), a common environmental contaminant, is widely used as an antifouling agent in paint. We previously reported that exposure of primary cortical neurons to TBT *in vitro* decreased the *α*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit glutamate receptor 2 (GluR2) expression and subsequently increased neuronal vulnerability to glutamate. Therefore, to identify whether GluR2 expression also decreases after TBT exposure *in vivo*, we evaluated the changes in GluR2 expression in the mouse brain after prenatal or postnatal exposure to 10 and 25 ppm TBT through pellet diets. Although the mean feed intake and body weight did not decrease in TBT-exposed mice compared with that in control mice, GluR2 expression in the cerebral cortex and hippocampus decreased after TBT exposure during the prenatal period. These results indicate that a decrease in neuronal GluR2 may be involved in TBT-induced neurotoxicity, especially during the fetal period.

Key words tributyltin; glutamate receptor 2; developmental neurotoxicity

Tributyltin (TBT), an organotin compound was widely employed as an antifouling agent in paints used for aquaculture nets and ships. Because of its irreversible effects on sexual abnormalities, known as imposex, in some female gastropods,[1] the use of TBT in paints is currently internationally restricted. Although the risk of acute environmental exposure to TBT has decreased, the issue of secondary exposure from contaminated sea food remains owing to its long-term environmental persistence and high bioaccumulation.[2,3] Some studies indicated that TBT-induced endocrine disruption was caused by inhibition of aromatase enzymes, which catalyze the conversion of androgen to estrogen.[4,5] Conversely, other studies reported that TBT-induced endocrine disruption in gastropods was caused by retinoid X receptor (RXR) activation because 9-*cis* retinoic acid (a well-known RXR ligand) induces gastropod imposex and TBT is a potential agonist of RXR.[6,7]

Ionotropic glutamate receptors, which are tetrameric ligand-gated ion channels, are essential to brain functions, such as learning and memory, and for neuronal growth, maturation, and synaptic plasticity. However, over-activation of glutamate receptors increases cytosolic Ca^{2+}, which triggers neuronal death. The *α*-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor is an ionotropic glutamate receptor and is composed of four subunits (GluR1 to GluR4). Its Ca^{2+} permeability is dependent on the glutamate receptor 2 (GluR2) subunit.[8] At steady state, most AMPA receptors contain GluR2 subunits in the cortical, hippocampal granule, and pyramidal neurons,[9] and are impermeable to Ca^{2+}. However, it is considered that decreases in neuronal GluR2 have been linked to neurotoxicity because neurons that contain GluR2-lacking AMPA receptors show high Ca^{2+} permeability and vulnerability to excitotoxicity.[8]

Organotin compounds, including TBT, are also known as neurotoxic compounds because they can permeate the blood–brain barrier (BBB).[10] The neurotoxic effects of TBT have been reported previously. The levels of neurotransmitters in the midbrain of TBT-administered mice changed[11] and the mice exhibited behavioral abnormalities.[12] Moreover, we previously reported neurotoxic events induced by TBT.[13–15] In particular, low-concentration TBT exposure decreased GluR2 expression and subsequently increased neuronal vulnerability to glutamate in primary cortical neurons.[16] However, additional research is needed to determine whether TBT exposure decreases GluR2 expression in mammalian brain. Thus, the aim of this study was to examine the effect of TBT on GluR2 expression *in vivo*.

MATERIALS AND METHODS

Materials TBT was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Pellet diets containing TBT were made by mixing TBT with commercial rodent diet, MF (Oriental Yeast, Tokyo, Japan)

Animals The present study was approved by the animal ethics committee of Hiroshima University. Mice (C57BL/6J Jcl) were housed at 20–26°C with 40–60% humidity on a 12-h light/12-h dark cycle with lights turned on at 8:00 am. Pregnant mice (4–5 months old) were fed pellet diets containing 10, 25 ppm TBT, and normal diets on gestation day (GD) 0 (the day of mucus plug formation was designated as GD 0) through postnatal day (PND) 20. After dams delivered pups on GD 20 (the day of birth was designated as PND 0), pups were exchanged between the TBT-treated dams and control dams on PND 1. In this way, those not prenatally exposed to TBT were raised by dams that were administered feed containing TBT and those that were prenatally exposed to TBT were raised by dams that were administered feed without TBT. Three groups of pups (control, postnatal TBT exposure,

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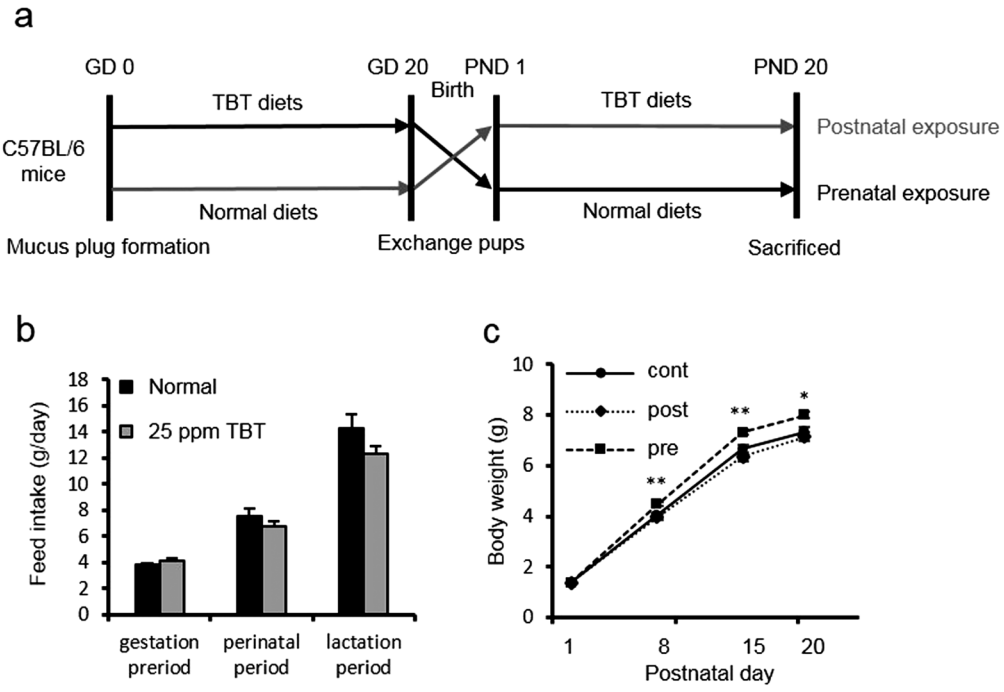

Fig. 1. Schematic of Experimental Design, Feed Intake, and Body Weight

(a) Experimental design schematic. (b) The mean daily intake of normal feed and feed containing 25 ppm TBT was measured during three periods: the gestation period (GD 3–GD 14), perinatal period (GD 11–PND 9), and lactation period (PND 6–PND 16). Data are expressed as mean±S.E.M. (n=3–4). (c) The mean body weight of control, postnatal 25 ppm TBT-exposed (post), and prenatal 25 ppm TBT-exposed (pre) pups at PND 1, 8, 15, and 20. Data are expressed as mean±S.E.M. (n=22–25) *p<0.05, **p<0.01 *vs.* control.

and prenatal TBT exposure) were sacrificed on PND 20. Tissues (cortex and hippocampus) were dissected, quickly frozen on dry ice, and stored at −80°C until further processing. The mean feed intake was measured during three periods: gestation period (GD 3–GD 14), perinatal period (GD 11–PND 9), and lactation period (PND 6–PND 16). The experimental design is shown in Fig. 1a.

Western Blotting Western blotting was performed as previously described.[17)]

Immunohistochemistry Immunohistochemistry was performed as previously described.[17)] Microscope slides were observed under a confocal laser scanning microscope (Olympus, FV-1000-D).

Statistics All experiments were performed at least thrice and representative data are shown. Data are expressed as mean±standard error of the mean (S.E.M.) Statistical evaluation of the data was performed with ANOVA followed by Tukey's test. A value of p<0.05 was considered statistically significant.

RESULTS

Feed Intake and Changes in Body Weight of Mice during the Experimental Period First, we measured the mean feed intake during three periods. The mean daily feed intake of normal diets and those containing 25 ppm TBT during the gestation period was 3.85 and 4.16 g/d, respectively; that during the perinatal period was 7.58 and 6.77 g/d, respectively; and that during the lactation period was 14.3 and 12.3 g/d, respectively (Fig. 1b). No significant difference in feed intake was observed between mice given normal feed and those given feed containing 25 ppm TBT during the experimental period.

Next, we measured the body weight of mice pups. On PND 1, the mean body weight was not significantly different between control, postnatal, and prenatal 25 ppm TBT-exposed pups. However, the mean body weight of prenatal 25 ppm TBT-exposed pups was significantly greater than that of the control pups on PND 8, 15, and 20 (Fig. 1c).

Effect of TBT on GluR2 Expression in Mouse Cerebral Cortex and Hippocampus The expression of all AMPA receptor subunits in the cerebral cortex and hippocampus was not significantly different between the prenatal and postnatal groups exposed to 10 ppm TBT (Figs. 2a–d). The expression of GluR2 in the cerebral cortex significantly decreased after prenatal exposure to 25 ppm TBT, but did not change after postnatal exposure to 25 ppm TBT (Figs. 2e, f). The expression of GluR1, GluR3, and GluR4 in the cerebral cortex was not altered by 25 ppm TBT exposure (Figs. 2e, f). As shown in Figs. 2g and h, the expression of GluR2 in the hippocampus significantly decreased after prenatal exposure to 25 ppm TBT, but did not change after postnatal exposure to 25 ppm TBT. In addition, GluR1 expression in the hippocampus modestly but significantly decreased after prenatal exposure to 25 ppm TBT (Figs. 2g, h). The expression of GluR3 and GluR4 was not altered by 25 ppm TBT exposure (Figs. 2g, h). These results suggested that prenatal exposure to 25 ppm TBT significantly decreased GluR2 expression in the mice cerebral cortex and hippocampus.

We immunostained neurons in cerebral cortex and hippocampas with GluR2 and anti-microtubule-associated protein 2 (MAP2) (a neuronal marker) antibodies to confirm the TBT-induced decrease in GluR2 expression. Neuronal GluR2 expression in cerebral cortex and hippocampus co-localized with MAP2 (Figs. 3a, b); furthermore, GluR2 levels co-localized with MAP2 were decreased after prenatal exposure to 25 ppm

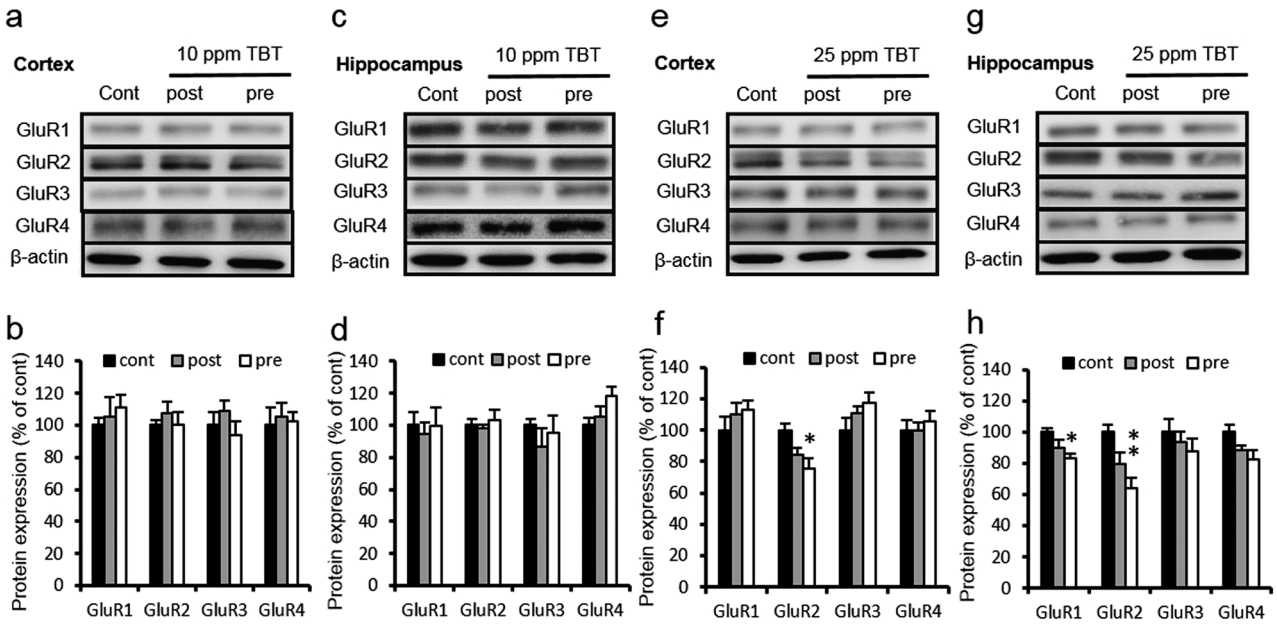

Fig. 2. TBT-Induced Changes in AMPA Receptor Subunit Expression in the Mouse Brain

GluR1, GluR2, GluR3, and GluR4 protein expression in the (a, e) cerebral cortex and (c, g) hippocampus was measured by Western blotting. (b, d, f, h) Quantitative GluR1, GluR2, GluR3, and GluR4 protein levels were analyzed with Image J software and corrected for β-actin protein levels. Data are expressed as mean±S.E.M. (n=5) *p<0.05, **p<0.01 *vs.* control.

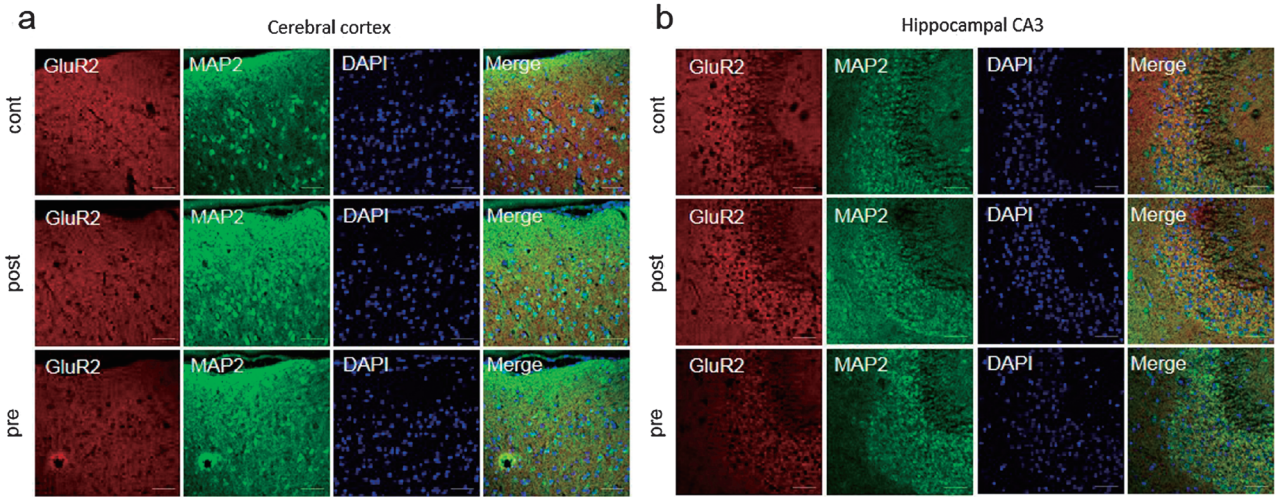

Fig. 3. Effect of TBT on Neuronal GluR2 Expression

Immunostaining was performed using a rabbit anti-GluR2 antibody (red) and mouse anti-MAP2 antibody (green) in the (a) cerebral cortex and (b) CA3 region of the hippocampus in mice. DAPI was used for nuclear staining (blue). Yellow or orange indicate co-localization of GluR2 and MAP2, respectively. Scale bar: 50 μm.

TBT (Figs. 3a, b), in accordance with the results in Figs. 2e–h.

DISCUSSION

Given the neurotoxic effects of exposure to TBT and the role of GluR2 in neurotoxicity, we evaluated the effect of TBT on GluR2 expression *in vivo*. Prenatal exposure of F0 mice to TBT through drinking water was reported to increase white adipose tissue depot weight, adipocyte number, and adipocyte size in F1 and subsequent generations. This effect resulted from epigenetic up-regulation of hepatic genes such as peroxisome-proliferator activated receptor γ (PPARγ), which is involved in lipid storage/transport, lipolysis, and lipogenesis.[18] Therefore, an increase in body weight following prenatal TBT exposure, as was observed in this study, may have been caused by epigenetic changes of genes involved in lipid accumulation.

In this work, we showed that prenatal TBT exposure decreased GluR2 expression in the mice brain (Figs. 2, 3). In general, GluR2 protein expression exhibits a sharp increase around birth and reaches a plateau around PND 20 in the mammalian brain.[19] Therefore, it is possible that TBT inhibited the GluR2 increase usually seen during the developmental period. Cooke *et al.* reported that TBT levels detected from TBT-postnatal exposed rat pups were markedly lower than those from TBT-prenatal exposed rat pups.[20] Given that pups were exposed to TBT through breast milk during postnatal exposure, the reason why significant GluR2 decrease was not

observed during postnatal exposure may be explained by low translation ratio of TBT into breast milk.

We previously reported that some environmental chemicals, such as lead, methoxyclor, fenvalerate, and perfluorooctane sulfonate (PFOS), decreased GluR2 expression and induced related neurotoxicity *in vitro*.[17,21–23] In addition, we have established a high-throughput screening system to identify the potential of chemicals to decrease GluR2, and some chemicals were detected as GluR2 decrease agents by this screening.[24] Among them, PFOS exposure during the fetal and neonatal periods decreased GluR2 expression in the animal brain, which resulted in enhanced neuronal vulnerability to kainic acid (a known neurotoxic glutamate analog).[17] In addition, GluR2 knockdown rats showed neuronal vulnerability to global ischemia[25] and systemic kainic acid injection.[26] These data suggested that a decrease in GluR2 is an important neurotoxic mechanism of chemicals and that various environmental chemicals have the potential to decrease GluR2.

In conclusion, our findings raise the possibility that a decrease in neuronal GluR2 expression may be involved in the mechanism of neurotoxicity induced by TBT, especially during the fetal period.

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Conflict of Interest The authors declare no conflict of interest.

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