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ORGAN TOXICITY AND MECHANISMS

Carbofuran causes neuronal vulnerability to glutamate by decreasing GluA2 protein levels in rat primary cortical neurons

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Abstract Glutamate receptor 2 (GluA2/GluR2) is one of the four subunits of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR); an increase in GluA2-lacking AMPARs contributes to neuronal vulnerability to excitotoxicity because of the receptor's high Ca^{2+} permeability. Carbofuran is a carbamate pesticide used in agricultural areas to increase crop productivity. Due to its broad-spectrum action, carbofuran has also been used as an insecticide, nematicide, and acaricide. In this study, we investigated the effect of carbofuran on GluA2 protein expression. The 9-day treatment of rat primary cortical neurons with 1 μM and 10 μM carbofuran decreased GluA2 protein expression, but not that of GluA1, GluA3, or GluA4 (i.e., other AMPAR subunits). Decreased GluA2 protein expression was also observed on the cell surface membrane of 10 μM carbofuran-treated neurons, and these neurons showed an increase in 25 μM glutamate-triggered Ca^{2+} influx. Treatment with 50 μM glutamate, which did not affect the viability of control neurons, significantly decreased the viability of 10 μM carbofuran-treated neurons, and this effect was abolished by pre-treatment with 300 μM 1-naphthylacetylspermine, an antagonist of GluA2-lacking AMPAR. At a concentration of 100 μM, but not 1 or 10 μM, carbofuran significantly decreased acetylcholine esterase activity, a well-known target of this chemical. These results suggest that carbofuran decreases GluA2 protein expression and increases neuronal vulnerability to glutamate toxicity at concentrations that do not affect acetylcholine esterase activity.

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Introduction

The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) is an ionotropic glutamate receptor widely distributed in the mammalian central nervous system (Hollmann and Heinemann 1994). AMPARs mediate fast synaptic transmission at excitatory synapses through Ca^{2+} permeation (Seeburg 1993). AMPARs are tetrameric ion channels composed of various combinations of four subunits: GluA1–GluA4 (Boulter et al. 1990; Fleck et al. 2003). Among these subunits, only GluA2 renders the receptor impermeable to Ca^{2+}; thus, GluA2-containing AMPARs are impermeable to Ca^{2+}, whereas GluA2-lacking AMPARs are permeable to Ca^{2+} (Hollmann et al. 1991; Verdoorn et al. 1991; Friedman et al. 2003). Ca^{2+} permeability through AMPARs determines synaptic plasticity and cell death associated with neurologic diseases and disorders (Liu and Zukin 2007). Therefore, the composition of these four subunits is crucial for AMPAR function.

We previously showed that long-term exposure to several environmental chemicals, including tributyltin, lead, methoxychlor, fenvalerate, and perfluorooctane sulfonate, decreases GluA2 protein expression and consequently increases neuronal vulnerability to glutamate stimulation and neuronal death (Nakatsu et al. 2009; Kotake 2012; Ishida et al. 2013; Umeda et al. 2016; Ishida et al. 2017). These studies suggest that decreased GluA2 protein expression can be used as an indicator of neurotoxicity. We previously established a high-throughput screening method based on AlphaLISA® technology (PerkinElmer, Inc.) to detect GluA2 protein expression levels as an index of neurotoxicity,

and 20 environmental chemicals at concentrations of 1 and 10 μM were tested using this screening method (Sugiyama et al. 2015). Our results identified 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate (carbofuran) as a candidate chemical that can decrease GluA2 protein expression.

Carbofuran, a carbamate pesticide, has been widely used to kill unwanted pests and insects in houses, gardens, and agricultural areas. Carbofuran is now banned in many countries due to the risks posed to humans and the environment; moreover, it has the potential to contaminate a variety of aquatic resources because of its solubility in water and moderately long half-life in soil (Yen et al. 1997; Agrawal and Sharma 2010). The presence of carbofuran and its metabolites in various environmental components, such as the soil and water, has been found due to indiscriminate and continuous applications (Sánchez-Brunete et al. 2003; Otieno et al. 2010; Jaiswal et al. 2016). In addition, carbofuran has been detected in human plasma samples (Whyatt et al. 2003; Petropoulou et al. 2006; Jaiswal et al. 2016).

Carbamate pesticides, including carbofuran, inhibit acetylcholine esterase (AChE) activity, which causes accumulation of endogenous acetylcholine and hyperactivity of cholinergic compounds in the autonomic nervous system. This change disturbs cholinergic synaptic transmission and causes paralysis. Thus, respiratory depression and pulmonary edema are the typical causes of death from poisoning by carbofuran. Carbofuran toxicity tends to be short duration because of the reversibility of its AChE inhibitory effect. Administration of carbofuran inhibited AChE activity in the rat brain by 0.5 h after dosing, and activity was restored to control levels by 6 h after dosing (Padilla et al. 2007). However, the non-cholinergic neurotoxicity caused by long-term carbofuran exposure remains unclear. The present study investigated the effect of long-term exposure to carbofuran in rat primary cortical neurons, focusing on the neurotoxicity triggered by decreased GluA2 protein expression.

Materials and methods

Materials

Eagle's minimal essential salt medium (MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Nichirei Bio-sciences (Tokyo, Japan). Horse serum (HS) was purchased from Gibco (Carlsbad, CA, USA). Alexa Fluor® 488-conjugated secondary antibody and 6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Life Technologies (Carlsbad, USA).

Carbofuran with a purity of 99.7%, D-(+)-glucose, dimethyl sulfoxide (DMSO), dipotassium hydrogenphosphate, $NaHCO_3$, phenylmethylsulfonyl fluoride (PMSF), and sodium dodecyl sulfate (SDS) were purchased from Wako (Osaka, Japan). Arabinosylcytosine, $MgCl_2 \cdot 6H_2O$, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), mouse anti-β-actin monoclonal antibody (AC-15), 1-naphthylacetylspermine (NAS), and trypan blue solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). KCl, KH_2PO_4, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris–HCl, nonidet P-40, ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate (DOC), 2-mercaptoethanol, and protease inhibitor cocktail were purchased from Nacalai Tesque (Kyoto, Japan). Mouse anti-GluA1 monoclonal antibody (MAB2263) and mouse anti-GluA2 monoclonal antibody (MAB397) were purchased from Millipore (Billerica, MA, USA). Rabbit anti-GluA3 monoclonal antibody (D47E3) and rabbit anti-GluA4 (D41A11) monoclonal antibody were purchased from Cell Signaling Technology Japan (MA, USA). Rabbit anti-N-cadherin (sc-7939) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Fura-2 AM, Pierce™ BCA Protein Assay Kit, and sulfo-NHS-LC-Biotin were purchased from Thermo Fisher Scientific K.K. (Waltham, Massachusetts, USA). Streptavidin agarose ultra-performance was purchased from Cosmo Bio (Tokyo, Japan). $CaCl_2$ and $MgSO_4$ were purchased from Kanto Chemical (Tokyo, Japan).

Cell culture

The following procedures were performed under sterile conditions. The present study was approved by the animal ethics committee of Hiroshima University. Primary cell cultures were obtained from the cerebral cortex of pregnant Slc:Wistar/ST rats at 18 days of gestation. The prefrontal cerebral cortex was dissected with a razor blade, and cells were plated on culture plates (3.4×10^5 cells/cm^2) in Eagle's MEM supplemented with 10% FCS, L-glutamine (2 mM), D-(+)-glucose (11 mM), $NaHCO_3$ (24 mM), and HEPES (10 mM). The cultures were maintained at 37 °C in an atmosphere of humidified 5% CO_2 in air for 10 days from 1 days in vitro (DIV) to 11 DIV. After 6 days in culture (1–7 DIV), the culture media were switched from MEM containing 10% FCS to MEM containing 10% HS for 4 days (7–11 DIV). Arabinosylcytosine (10 μM) was added to inhibit the proliferation of non-neuronal cells at 6 DIV, and the cell cultures were used for experiments at 11 DIV.

Treatment with chemicals

Carbofuran or DMSO as a vehicle was continuously added to the culture medium for 9 days from 2 DIV (24 h after preparation) to 11 DIV. Media containing carbofuran or DMSO was replaced every 2 days.

Sample preparation

Cortical neurons were assessed at 11 DIV. Protein extraction was completed using a previously published method (Umeda et al. 2016; Miyara et al. 2016). Cells were washed with ice-cold PBS and lysed in TNE buffer containing 50 mM Tris–HCl, 1% Nonidet P-40, 20 mM EDTA, 1% protease inhibitor cocktail, 1 mM sodium orthovanadate, and 1 mM PMSF. The mixture was rotated for 30 min at 4 °C and then centrifuged at 15,000 rpm for 25 min. The supernatants were removed to quantify the amount of total protein with a Pierce™ BCA Protein Assay Kit and GluA2 protein expression using western blotting.

Western blotting

Cell extracts were added to a sample buffer containing 100 mM Tris–HCl, 4% SDS, 20% glycerol, and 0.004% bromophenol blue. Proteins were denatured with 5% 2-mercaptoethanol at 95 °C for 3 min. Proteins were separated using SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. After transfer, membranes were blocked for 1 h with a blocking buffer containing 5% skim milk and then incubated with anti-GluA1 (1:3000), anti-GluA2 (1:2000), anti-GluA3 (1:3000), anti-GluA4 (1:1000), anti-N-cadherin (1:2000), and anti-β-actin (1:20,000) antibodies overnight at 4 °C. After incubation with secondary antibodies at room temperature for 1 h, proteins were detected with an enhanced chemiluminescence detection system (Chemi-Lumi One L; Nacalai Tesque, Kyoto, Japan). Quantitative analyses were performed using digital imaging software (Image J; NIH, Bethesda, MD, USA). Total GluA2 protein levels were normalized to β-actin.

Immunocytochemistry for GluA2 protein on the cell surface

GluA2 protein localized in cell surface membrane was stained by labeling extracellular N-terminal domain of GluA2 without membrane permeabilization using our previously reported method which is confirmed to selectively stain surface proteins (Umeda et al. 2016). Cells were plated on poly-D-lysine-coated 4-well chamber slides (BD Bioscience) and incubated overnight. After exposure to 10 μM carbofuran for 9 days, cells were washed with PBS(−) and fixed with 4% paraformaldehyde in PBS(−) for 15 min at room temperature. Slides were washed with PBS(−) and then blocked with 8 drops of Image-iT® FX Signal Enhancer (Life Technologies) for 1 h. Surface proteins were incubated with an anti-GluA2 antibody (1:200), which recognizes GluA2 N-terminal domain on the external surface of the cellular membrane, in PBS(−) overnight at 4 °C. The slides were washed 3 times with PBS(−) and incubated with Alexa Fluor® 488-conjugated secondary antibody (1:800) for 1 h at room temperature in the dark. The slides were further washed 3 times with PBS(−) and incubated with 600 nM DAPI in PBS(−) for 5 min. The slides were washed 2 more times with PBS(−) and mounted in Prolong® Diamond antifade reagent (Life Technologies). After an overnight incubation, surface GluA2 expression was evaluated using a confocal laser scanning microscope (LSM5 PASCAL, Carl Zeiss).

Surface protein extraction

Cell surface GluA2 was quantified using the method described by Liu et al. (2010). Cell surface proteins were labeled with biotin, and whole proteins were separated into cell surface and intracellular fractions using streptavidin beads bound to biotin-surface protein complex. Cells were washed 3 times with PBS containing 1 mM $CaCl_2$ and 1.3 mM $MgCl_2$ (PBS (+)) on ice, and incubated with 1 mg/mL sulfo-NHS-LC-biotin in PBS (+) for 20 min at 4 °C to bind cell surface proteins. Next, cells were washed with 100 mM glycine on ice to quench the biotin reaction and lysed in 500 μL lysis buffer containing 0.5% DOC, 0.5% Nonidet P-40, 0.2% SDS, 1% protease inhibitor cocktail, 1 mM sodium orthovanadate, and 1 mM PMSF in PBS (+). The lysates were centrifuged at 13,000*g* for 15 min at 4 °C. An aliquot (100 μL) of the supernatant fraction was removed to measure protein amount. The remaining supernatant fraction (400 μL) was incubated with 30 μL streptavidin agarose beads overnight at 4 °C to promote the binding of streptavidin beads and biotin-conjugated surface proteins. The beads were centrifuged at 500*g* for 5 min at 4 °C. Next, the removed supernatants were assessed for intracellular protein expression. The beads bound to surface proteins were washed three times with lysis buffer before being resuspended in 35 μL SDS sample buffer and boiled at 95 °C for 10 min. GluA2 expression was detected with western blotting using surface and intracellular protein fraction extracts and anti-GluA2 antibody.

Measurement of intracellular Ca^{2+} concentration

Ca^{2+} measurements were performed using previously described methods (Nakatsu et al. 2007). Rat primary cortical neurons on poly-D-lysine-coated 8-well chamber slides (BD Bioscience) were loaded with 5 μM Fura-2 AM for 30 min in HEPES-buffered salt solution (HBSS) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES. Slides were washed 3 times with HBSS, and then changes in intracellular Ca^{2+} concentration were evaluated by measuring the fluorescence intensity ratio (340/380 nm). Next, 25 μM glutamate was added to neurons in HBSS 1 min

after the start of measurement. The video image output was digitized by an Argus-100/HiSCA (Hamamatsu Photonics, Hamamatsu, Japan). Each point represents the mean of 30 cells.

Evaluation of neuronal death

Neurotoxicity was quantified with a trypan blue dye exclusion assay (Sugiyama et al. 2015; Umeda et al. 2016). After exposure to carbofuran or DMSO, neurons were stimulated with 50 μM glutamate for 24 h, stained with a 1.5% trypan blue solution for 10 min, fixed with 10% formalin for 2 min, and rinsed with physiological saline. Stained cells were regarded as dead, and unstained cells were regarded as alive. Cell viability was calculated as the percentage ratio of unstained cells to total cells counted. Over 200 cells per culture dish were counted at random.

Measurement of AChE activity

AChE activity was measured using a spectrophotometric analysis based on Ellman's method with some modifications (Ellman et al. 1961; Willig et al. 1996). The method uses DTNB to quantify the thiocholine produced from the hydrolysis of acetylthiocholine by AChE. Rat cortical neurons were dissected and cultured for 11 days, and proteins were extracted at the end of cultivation. A total of 140 μL of 0.27 mM DTNB in potassium phosphate buffer and 10 μL carbofuran (0.1–100 μM), and 10 μL protein extracts were added to the 96-well plate on ice and mixed. Next, acetylthiocholine iodide as a substrate was quickly added to the plate. After incubation at 37 °C for 10 min, the yellow absorbance of 5-thio-2-nitrobenzoate anion from DTNB by thiocholine was measured at 415 nm using a multimode plate reader (EnSpire™; PerkinElmer, Inc.).

Statistics

All experiments were replicated and representative data are shown. Data are expressed as the mean ± SD. Statistical analyses of the data were performed using an ANOVA followed by a Tukey's test. P values of <0.05 were considered statistically significant.

Results

Exposure to carbofuran decreases GluA2 protein expression without affecting other AMPAR subunits

To examine the effect of carbofuran on GluA2 protein expression levels, rat primary cortical neurons were exposed to 0.1–100 μM carbofuran for 9 days from 2 DIV to 11 DIV. GluA2 protein expression was measured using western blotting. Carbofuran significantly decreased GluA2 protein expression at the lowest tested concentration of 1 μM (Fig. 1a). Concentrations of 1 μM and 10 μM carbofuran did not significantly affect the other AMPAR subunits GluA1, GluA3, and GluA4 (Fig. 1b). Thus, the exposure of rat primary cortical neurons to carbofuran specifically decreases GluA2 expression.

Exposure to carbofuran decreases GluA2 protein expression in the cell surface membrane

AMPARs cycle between the inside of the cell and the surface membrane to modulate synaptic strength (Bredt and Nicoll 2003; Czöndör and Thoumine 2013; Henley and Wilkinson 2013). We examined the effect of exposure to 10 μM carbofuran for 9 days on cell surface GluA2 protein expression in rat primary cortical neurons using immunocytochemistry without membrane permeabilization. Carbofuran decreased surface GluA2 protein expression (Fig. 2a). We confirmed this result by measuring GluA2 protein levels in both cell surface membrane and intracellular fractions. Exposure to 10 μM carbofuran decreased GluA2 protein expression not only in intracellular fraction, but also in the cell surface fraction (Fig. 2b). These results suggest that the exposure to carbofuran decreases overall GluA2 protein levels.

Exposure to carbofuran increases glutamate-induced Ca^{2+} influx

We predicted that carbofuran increases Ca^{2+} permeability because GluA2-lacking AMPARs are known to be Ca^{2+} permeable. Therefore, we measured glutamate-induced intracellular Ca^{2+} influx. Rat primary cortical neurons were treated with 10 μM carbofuran for 9 days, and glutamate-induced intracellular Ca^{2+} influx was measured by monitoring the fluorescence intensity of Fura-2 AM. Ca^{2+} influx during the 4 min after treatment with 25 μM glutamate increased in 10 μM carbofuran-treated neurons when compared with control neurons (Fig. 3a). The area under the curve of the Fura-2 AM fluorescence ratio showed that carbofuran significantly increased glutamate-induced Ca^{2+} influx (Fig. 3b).

Exposure to carbofuran induces neuronal vulnerability to glutamate toxicity

Ca^{2+} homeostasis is crucial for neuronal survival. Therefore, we examined whether carbofuran-induced decreases in GluA2 protein expression lead to neuronal death through excessive Ca^{2+} influx. Rat primary cortical neurons were treated with 10 μM carbofuran for 8 days from 2 DIV to 10 DIV, and neuronal vulnerability was evaluated by measuring cell viability at 11 DIV after a

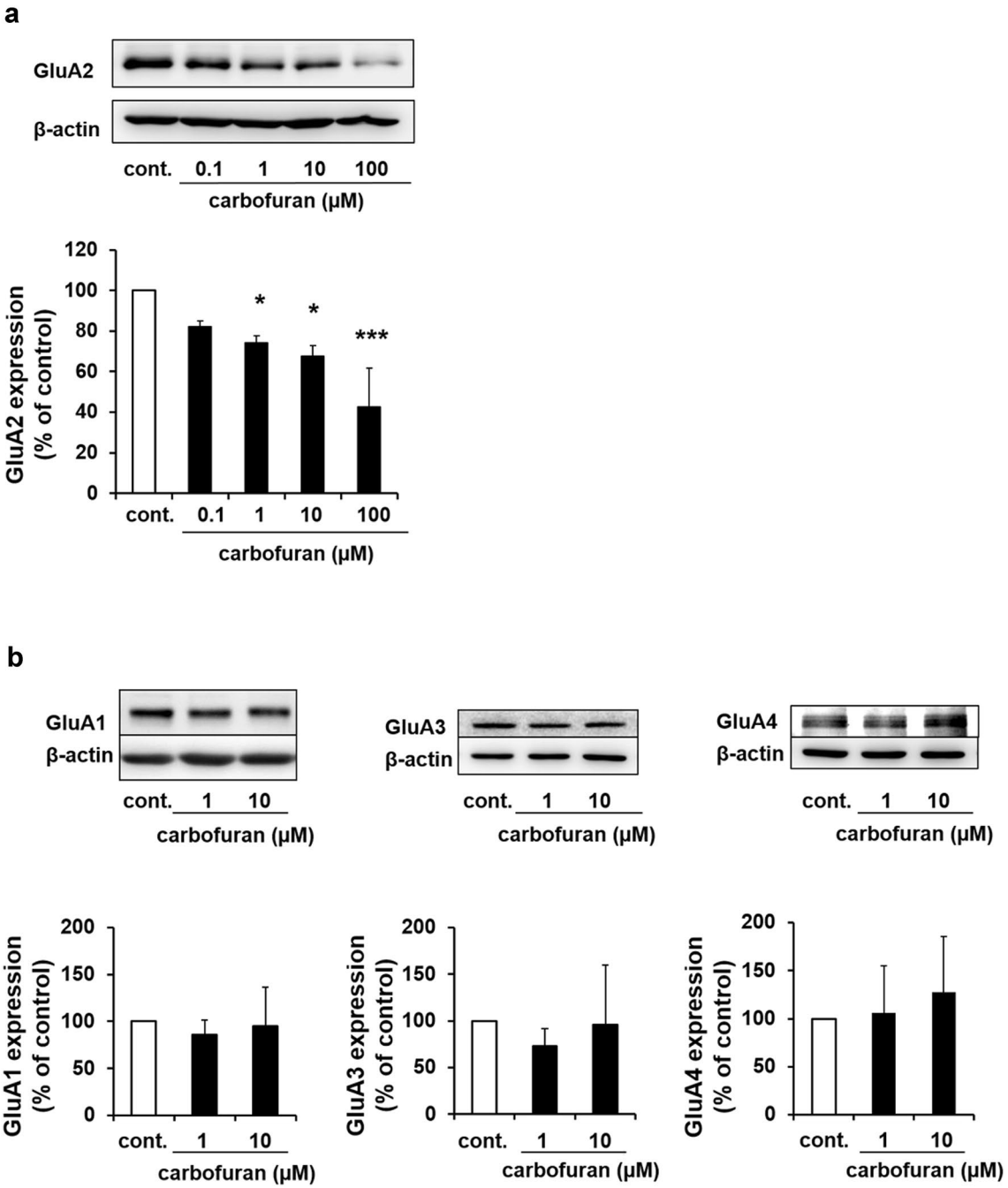

Fig. 1 Carbofuran-induced changes in the protein expressions of GluA2 and the other AMPAR subunits in rat primary cortical neurons. **a** Cortical neurons were exposed to DMSO (control) or 0.1–100 μM carbofuran for 9 days from 2 days in vitro (DIV) to 11 DIV. GluA2 protein expression was detected with western blotting. **b** Cortical neurons were exposed to DMSO (control) or 1 μM or 10 μM carbofuran for 9 days, and then AMPAR subunit, i.e., GluA1, GluA3, and GluA4, protein expression was detected with western blotting. Quantitative analysis was performed with Image J software, and subunit protein levels were normalized against β-actin. Data are expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control

50 μM glutamate treatment for the last 24 h of culture. Glutamate stimulation significantly decreased cell viability in 10 μM carbofuran-treated neurons but not control neurons (Fig. 4). The 30-min pre-treatment with 300 μM NAS, a selective antagonist of GluA2-lacking AMPARs, completely suppressed glutamate-induced neuronal death in carbofuran-treated neurons (Fig. 4). These results indicate that exposure to carbofuran induces neuronal

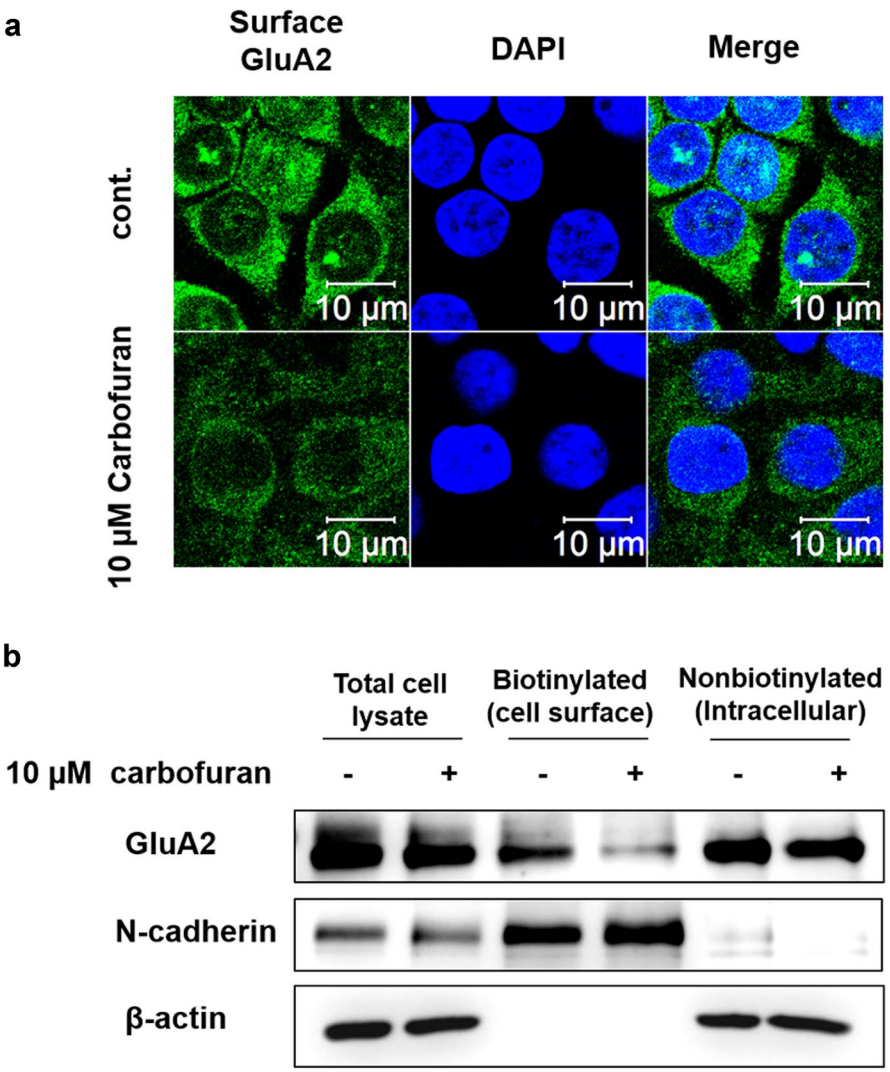

Fig. 2 Carbofuran-induced decrease in surface GluA2 protein expression in rat primary cortical neurons. Cortical neurons were exposed to DMSO (control) or 1 or 10 μM carbofuran for 9 days from 2 DIV to 11 DIV. **a** Surface GluA2 was detected with immunocytochemical staining (*green*). Nuclei were labeled with DAPI (*blue*). *Scale bars* 10 μm. **b** Surface proteins were biotinylated and separated in cell surface and intracellular fractions and total cell lysates. GluA2 protein levels in each fraction were assessed by western blotting. N-cadherin and β-actin were used as a cell surface marker and a cytosol marker, respectively (colour figure online)

vulnerability to glutamate toxicity via decreased GluA2 protein expression.

Effect of carbofuran on AChE activity

Carbofuran inhibits AChE activity. Thus, we added 0.1–100 μM carbofuran to protein mixtures extracted from 11 DIV rat primary cortical neurons. AChE activity was measured with the Ellman method. Carbofuran significantly decreased AChE activity at a concentration of 100 μM but not 0.1 or 10 μM (Fig. 5).

Discussion

We investigated the neurotoxicity of carbofuran-induced decreases in GluA2 protein expression in rat primary cortical neurons. We first confirmed that carbofuran decreases GluA2 protein expression in a concentration-dependent manner using western blotting (Fig. 1a). The mechanism behind the decrease in GluA2 protein expression by carbofuran remains unclear. The transcription of AMPAR subunits is regulated by several transcription factors, such as Sp1, nuclear respiratory factor-1 (NRF-1), and RE1-Silencing transcription factor (REST) (Myers et al. 1998). Sp1 enhances GluA2 promotor activity, whereas REST represses its activity (Myers et al. 1998; Huang et al. 1999). Furthermore, each AMPAR subunit GluA1-GluA4 is differentially regulated by specific transcriptional factors. For example, NRF-1 selectively binds to the GluA2 promotor (Dhar et al. 2009). Considering that carbofuran specifically decreased GluA2 (Fig. 1b), it is possible that carbofuran inhibits the GluA2 transcriptional process by affecting NRF-1 protein levels. GluA1,3,4 expressions did not significantly increase by carbofuran, but we have previously reported a tendency that their expressions were increased by tributyltin and perfluorooctane sulfonate instead of GluA2 decrease (Nakatsu et al. 2009; Ishida et al. 2017). Therefore, the compensatory

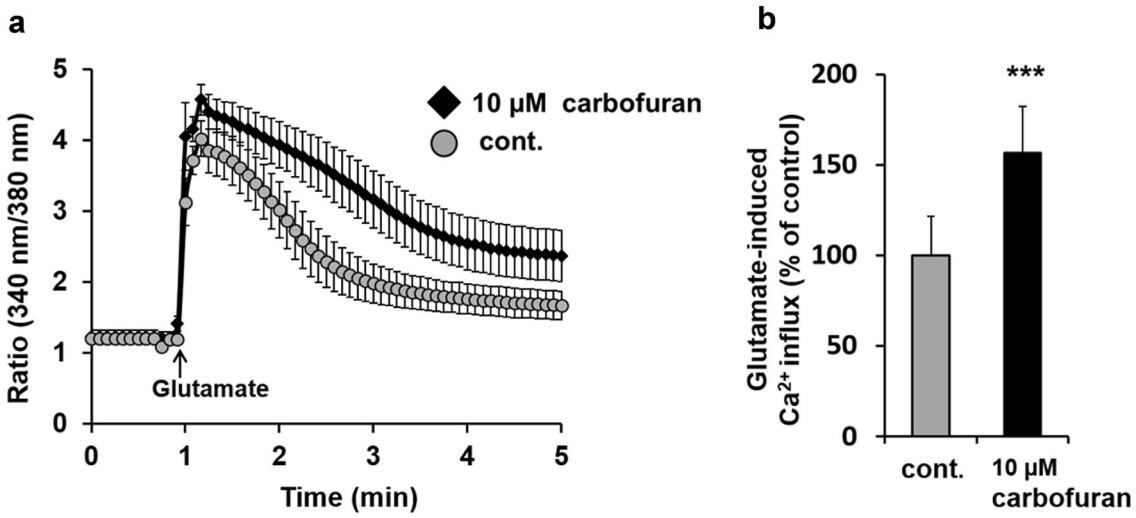

Fig. 3 Effect of long-term exposure to 10 μM carbofuran on glutamate-induced Ca^{2+} influx. Cortical neurons were exposed to DMSO (control) or 10 μM carbofuran for 9 days from 2 DIV to 11 DIV. Neurons were stimulated with 25 μM glutamate at 1 min after the start of measurement. **a** Changes in intracellular Ca^{2+} concentrations were evaluated by measuring the fluorescence intensity ratio (340/380 nm) using Fura-2 AM. **b** Changes in ratios were estimated. Data are expressed as the mean ± SD ($n = 30$). ***$P < 0.001$ versus control

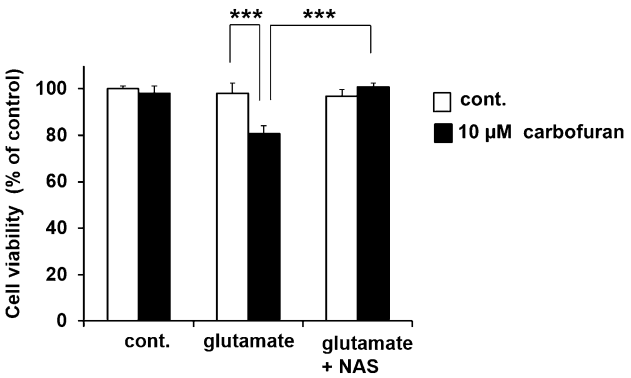

Fig. 4 Effect of long-term exposure to 10 μM carbofuran on glutamate toxicity. Cortical neurons were exposed to DMSO (control) or 10 μM carbofuran for 8 days from 2 DIV to 10 DIV. Next, 30 min after the pre-treatment with H_2O (control) or 300 μM NAS, neurons were exposed to 50 μM glutamate for 24 h from 10 DIV to 11 DIV. Cell viability was measured using a trypan blue assay at 11 DIV. Data are expressed as the mean ± SD ($n = 3$). ***$P < 0.001$

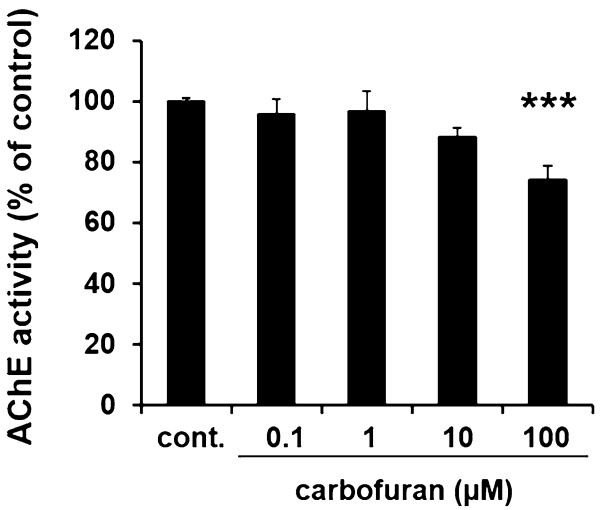

Fig. 5 Carbofuran-induced AChE inhibition in rat primary cortical neurons. Carbofuran (0.01–100 μM) was applied to a protein mixture extracted from 11 DIV rat primary cortical neurons. AChE activity was determined using the absorption intensity of DTNB adducts. Data are expressed as the mean ± SD ($n = 3$). ***$P < 0.001$ vs. control

mechanisms are expected though we do not know the precise mechanisms. It is reported that the loss of GluA2 does not alter AMPAR number, clustering, or distribution (Iihara et al. 2001), and thus decrease in GluA2 may not change overall receptor number in this study. We speculate that other GluA subunits compensate for GluA2 decrease to maintain overall receptor number.

We also determined that carbofuran decreased surface GluA2 protein expression (Fig. 2a, b). The density of AMPARs in the cell surface membrane is regulated by a dynamic balance between biosynthesis, export to the plasma membrane, endocytosis, and endosomal recycling. Under basal conditions, AMPARs shuttle continuously between intracellular pools, which include the majority of AMPARs (60–70%), and the surface membrane, which include the remaining AMPARs (30–40%) that function as transmembrane ion channels (Bredt and Nicoll 2003; Czöndör and Thoumine 2013). Therefore, it is possible that carbofuran indirectly decreases surface GluA2 protein levels by

reducing the reserved GluA2 in intracellular pools. Each AMPAR subunit is differentially delivered to the synapse membrane based on their cytoplasmic C-terminal tails (Passafaro et al. 2001) and the binding of several trafficking protein partners, including accessory and scaffolding proteins (Dong et al. 1997; Srivastava et al. 1998; Craven and Bredt 1998; Osten et al. 2000). Thus, the carbofuran-induced decrease in GluA2 at the surface could be due to modulation of these factors.

Our study showed that carbofuran increases glutamate-induced Ca^{2+} influx (Fig. 3). Among the four AMPAR subunits, only GluA2 renders AMPARs permeable to Ca^{2+}. The critical residue controlling Ca^{2+} permeability of GluA2 is in the pore loop region. The pore is occupied by a glutamine residue in GluA1, GluA3, and GluA4; whereas, in GluA2 the pore is occupied by an arginine residue. The voltage-dependent block by the presence of arginine in the pore loop region of GluA2 inhibits Ca^{2+} permeability (Bowie and Mayer 1995; Liu and Zukin 2007). Considering that the number of GluA2 subunits in the AMPAR complex shows a dose-dependency for Ca^{2+} permeability (Washburn et al. 1997; Isaac et al. 2007), it is possible that the carbofuran-induced decreases in GluA2 at the cell surface leads to neurons that are highly permeable to Ca^{2+}.

We showed that carbofuran treatment increased glutamate-induced neuronal death (Fig. 4). Several lines of evidence indicate that Ca^{2+} permeation through GluA2-lacking AMPARs is crucial in cell death. For example, knockdown of GluA2 induces cell death in hippocampal neurons in young rats during a specific postnatal period, i.e., when GluA2 expression peaks during development and glutamatergic inputs are maturing (Friedman and Velísková 1998). Downregulation of GluA2 and brief ischemia in rats synergistically caused neuronal death in the hippocampus (Oguro et al. 1999). In the present study, similar to the downregulation of GluA2, the cell viability of carbofuran-treated neurons decreased after stimulation of non-toxic amounts of glutamate. Furthermore, this effect was abolished by treatment with NAS (Fig. 4). These results suggest that decreased GluA2 in AMPARs is responsible for the carbofuran-induced vulnerability to glutamate. Exposure to 10 μM carbofuran caused GluA2 reduction and increased vulnerability to glutamate in neurons (Figs. 1a, 4), whereas it did not affect AChE activity in a protein mixture extracted from cultured neurons (Fig. 5). The major toxicity of carbofuran is the inhibition of AChE activity at synaptic junctions. Carbofuran readily passes the blood–brain barrier and quickly shows maximal inhibition of AChE activity. AChE activity returns to normal levels within a matter of hours, except for severe cases (Padilla et al. 2007). Therefore, studies on the non-cholinergic toxicity induced by long-term exposure to carbofuran are less well studied. The present study suggests that lower concentrations of carbofuran decrease GluA2 protein level without AChE activity inhibition, thus raising the possibility that carbofuran decreases GluA2 protein expression independently of AChE. In fact, we previously showed that AChE inhibitors which do not affect GluA2 protein expression exist (Sugiyama et al. 2015).

In summary, the present study demonstrated that carbofuran decreases GluA2 protein expression and leads to increased neuronal vulnerability to glutamate. We showed a novel neurotoxicity of carbofuran, but also highlighted the possibility that decreased GluA2 expression is a highly sensitive marker of neurotoxicity. Identification of other chemicals that decrease GluA2 expression and assessment of their neurotoxicity are important for the protection of human health.

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Compliance with ethical standards

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the animal ethics committee of Hiroshima University. This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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