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ORIGINAL ARTICLE

Acute and Chronic Exposure of Toluene Induces Genotoxicity in Different Regions of the Brain in Normal and Allergic Mouse Models

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Abstract

Toluene is a widely used industrial organic solvent and is ubiquitous in our environment. The neurobehavioral and neurotoxic effects of toluene are well recognized; however, its genotoxicity is still under discussion. Toluene biotransformation leads to the generation of reactive oxygen species that cause oxidative stress and DNA damages. Individuals with different immunogenetic backgrounds have different sensitivities to toxic chemical exposure. Previous studies have suggested that allergic stimulation may influence the threshold for toluene sensitivity due to the modulation of neurotrophin-related genes. Therefore, we aimed to investigate toluene-induced genotoxicity in different brain regions following acute and chronic exposure in vivo and to further examine whether allergic stimulation may influence the sensitivity to toluene-induced genotoxicity. In this present study, we found that exposure of toluene induced oxidative DNA damages resulting in genotoxicity in different brain regions including cortex, cerebellum, and hippocampus using comet assay. Higher genotoxicity induced by toluene was observed in the hippocampus of control mice compared to OVA-immunized mice. These results provide evidence that toluene-induced genotoxicity may contribute to its neurotoxicity in different immunogenetic individuals.

Keywords Toluene · Genotoxicity · Acute and chronic exposure · Hippocampus · BDNF

Introduction

Toluene (methylbenzene) is an aromatic hydrocarbon and a ubiquitous solvent used for industrial purposes and in a number of commercial products, such as cosmetics, inks, adhesive, paints, and glues (ATSDR 2000). It is the most widely abused inhaled volatile substance since toluene produces psychoactive effects when intentionally inhaled in pure form or from numerous commercial products (Cruz et al. 2014). As a result, exposure of toluene from numerous household products, occupational settings, or through inhalant abuse is increasing. The primary target of toluene is thought to be the central nervous system, and both acute and chronic effects are recognized (EPA 2005). In high doses, toluene has effects similar to other volatile substances such as psychomotor damage, excitation and later inhibition of locomotor activities, loss of the standing reflex, and sedation (Batis et al. 2010; Callan et al. 2017b). Toluene induces these effects by affecting the GABAergic, glutamatergic, serotonergic, and dopaminergic pathways (Callan et al. 2017a; Meydan et al. 2012). Toluene leads to apoptotic neurodegeneration in the cerebellum and hippocampus (Ladefoged et al. 2004). The occupational exposure limit for toluene is 20 ppm in the USA (ACGIH 2007). Exposure to low concentrations of toluene leads to persistent deficits in spatial learning and memory function in rat models (von Euler et al. 2000). Chronic toluene addiction is known to cause atrophy in the substantia alba and is associated with clinical outcomes of atrophy (Aydin et al. 2002). While the neurobehavioral and neurotoxic effects of toluene have been thoroughly examined, the mechanisms whereby toluene exerts its effects in the brain are not fully understood.

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Individuals with different immunogenetic backgrounds have different sensitivities to toxic chemical exposure. Previous studies have reported that ovalbumin (OVA) immunization may enhance the sensitivity to toluene exposure by modulating NMDA receptor subunit expression in the olfactory bulb of the allergic mouse model (Win-Shwe et al. 2010a). They also found that low-level toluene exposure may induce the upregulation of neurotrophin-related gene expression in the mouse hippocampus, depending on the mouse strain and that allergic stimulation in sensitive strains may decrease the threshold for sensitivity to a lower exposure level (Win-Shwe et al. 2010b). Neurotrophins are a group of structurally related polypeptides that support the survival, differentiation, and maintenance of neuronal populations expressing appropriate high-affinity neurotrophin receptors (Lu et al. 2005). Neurons in the hippocampus are maintained by neurotrophins, such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the tyrosine kinase (Trk) family of neurotrophin receptors. Neurotrophins and their related receptors have been identified as targets for neurotoxicants and are known to play a role in bidirectional signaling between cells of the immune and nervous systems (Betancourt et al. 2006; Kidd et al. 2008). It has been suggested that toluene exposure affects the function of the hippocampus by modulating neurotrophin-related genes and signaling pathways and that allergic stimulation as an immune stressor may influence the threshold for sensitivity (Win-Shwe and Fujimaki 2010).

Toluene is not classified as a carcinogenic solvent, and its genotoxicity is still under discussion (EPA 2005; IARC 1999). One pathway of toluene biotransformation leads to the formation of toluene epoxides, which may generate reactive oxygen species (ROS) and can cause oxidative stress and DNA damages (Kodavanti et al. 2011; Murata et al. 1999). DNA oxidation has potential genotoxic consequences (Martinez-Alfaro et al. 2010) and is implicated in the pathogenesis of several diseases (Costa et al. 2006), including cancer and neurodegenerative disorders (Marlatt et al. 2008). Previous studies have shown that in vivo exposure to various doses of toluene (0.5, 1.0, and 1.5 g/kg ip) elicited an elevation of ROS generation in different regions of the brain as well as in the blood. Among the brain regions, the hippocampus had the highest levels of induced ROS (Mattia et al. 1993). Furthermore, a significant increase in oxidative DNA damages occurred in lymphocytes from rats exposed to thinner fumes (toluene is the principal component of most thinners) compared to lymphocytes from control rats (Martinez-Alfaro et al. 2006). In addition, correlation of blood toluene levels and genetic damages in leukocytes of industrial painters from Rio Grande do Sul, Brazil, showed that low levels of toluene exposure can cause genetic damage, which is related to oxidative stress, age, and time of exposure (Moro et al. 2012).

Therefore, we hypothesized that toluene may cause oxidative DNA damages in brains resulting in genotoxicity and finally contributing to its neurotoxicity. Further, allergic stimulation as an immune stressor may influence the threshold for sensitivity to toluene-induced genotoxicity. Herewith, this present study was designed to investigate toluene-induced genotoxicity in different brain regions following acute and chronic exposure in vivo. Furthermore, we used OVA as a stressor to investigate whether exposure to low levels of toluene, in combination with OVA, might also affect neurotrophin-related gene expression and toluene genotoxicity.

Materials and Methods

Experimental Animals Random bred male Balb/c mice, 7 weeks of age, were supplied by the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, R.O.C. The animals were acclimatized for at least 1 week before the start of experiments, and mice were housed in groups of five per cage in an air-conditioned room (24 ± 1 °C) on a 12-h light/dark cycle (06:00–18:00-h light) and had free access to food and water. The use of animals has been approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taipei, Taiwan, R.O.C. All experiments were performed in accordance with the approved guidelines.

Exposure of Toluene For acute exposure of toluene, 27 mice were injected with toluene (CAS # 108-88-3) (5 or 15 g/kg, Sigma, Darmstadt, Germany, $n = 9$ for each dose) or corn oil as control ($n = 9$) intraperitoneally and sacrificed after 2 h for collection of brain, blood, and liver samples (Fig. 1a). For chronic exposure of toluene, 24 mice were nose-exposed with toluene vapor (25 ppm, $n = 12$) or filtered air ($n = 12$) for 4 weeks (Fig. 1b). Toluene vapor was generated using Dynacal Diffusion vials (VICI Metronics Inc., USA) diluted with clean filtered air to achieve the desired gas concentrations and then introduced into a stainless steel and glass chamber as described previously (Fig. 1c) (Ishidao et al. 2000). Air in the chamber was sampled periodically from the sampling port, and the vapor concentration was monitored by a gas chromatography mass spectrometer (Agilent 6890N, USA) equipped with a flame ionization detector (FID). The average levels of the control and 25 ppm toluene treatment were 0.01 ± 0.001 ppm and 25.1 ± 0.1 ppm, respectively. The airflow rate through the chamber housing the experimental animals was 20 L × min^{-1}. The concentrations of toluene were constant, irrespective of the sampling port location. Each group of mice ($n = 6$) was exposed to a filtered air control (0 ppm) or toluene for 6 h (from 10:00 to 16:00 h) per day, 5 days per week for 6 weeks in a nose-only exposure chamber. The allergic model mice were

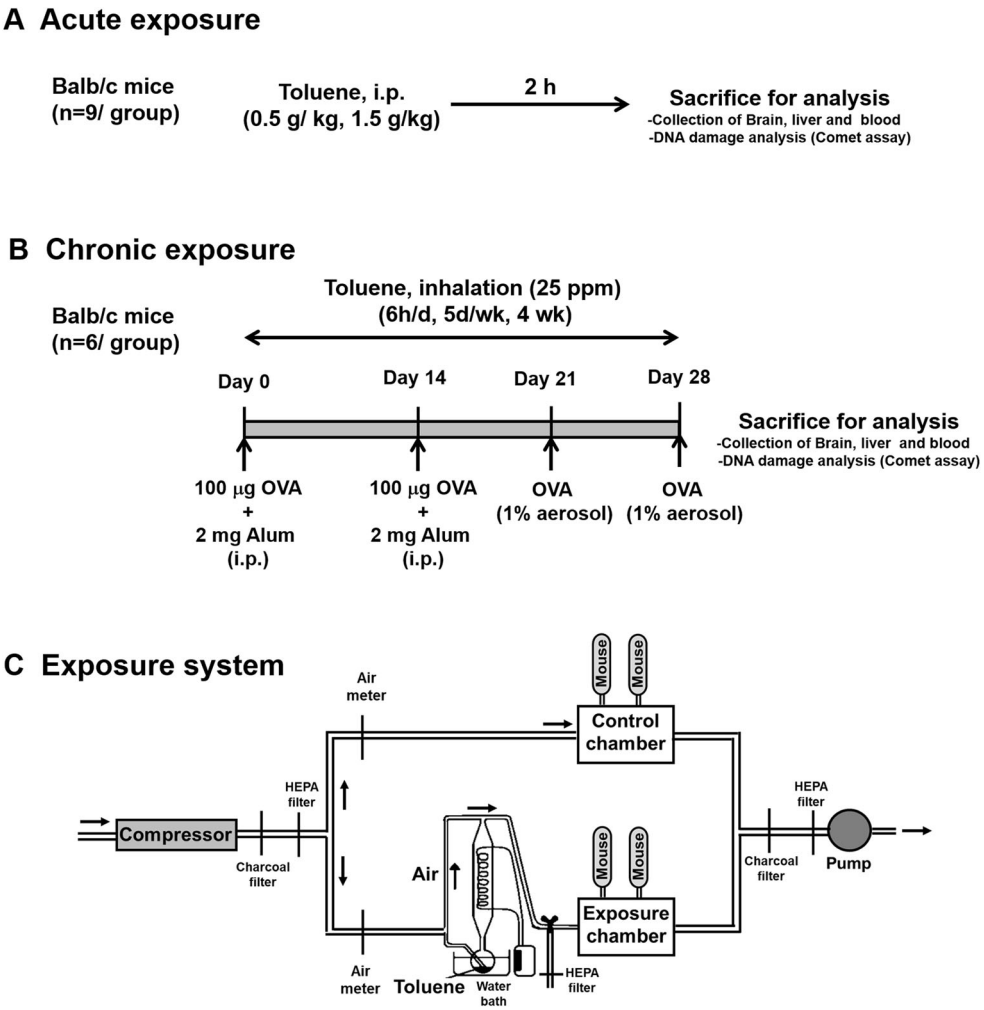

Fig. 1 Experimental design of toluene exposure and allergic stimulation. For acute exposure, Balb/c mice were injected with a control (corn oil, $n = 9$) or toluene (5 or 15 g/kg, $n = 9$ for each dose) intraperitoneally and sacrificed for collection of brain, liver, and blood samples 2 h later (**a**). For chronic exposure of toluene, Balb/c mice were exposed to either a filtered air control (0 ppm, $n = 12$) or toluene (25 ppm, $n = 12$) for 6 h per day, 5 days per week for 4 weeks. One day following the final toluene inhalation, the mice were sacrificed for collection of brain and blood samples (**b**). To detect whether the allergic condition affects toluene exposure, mice were immunized with a control (normal saline, $n = 6$) or ovalbumin (OVA, $n = 6$) on days 0, 14, 21, and 28 approximately 1 h before toluene exposure. These mice were injected with 100 μg of OVA plus 2 mg of alum intraperitoneally on days 0 and 14, and each of these mice was then challenged with nebulized OVA as a booster on days 21 and 28 during the exposure period. Design of toluene exposure system was shown as (**c**)

given OVA (Sigma, Darmstadt, Germany) immunization at days 0, 14, 21, and 28 approximately 1 h before toluene exposure. These mice were injected with 100 μg OVA plus 2 mg aluminum hydroxide (Alum, Thermo, USA) intraperitoneally on days 0 and 14. Each of these mice was then challenged with nebulized OVA as a booster on days 21 and 28 during the exposure period as described previously (Fig. 1b).

Measuring the Anti-OVA IgG1 Titer in Plasma Blood was sampled from the tail of the immunized mice subjected to OVA sensitization and challenge. The anti-OVA IgG1 antibodies in the plasma were measured by ELISA using an Anti-Ovalbumin IgG1 (mouse) ELISA Kit (Cayman Chemical, USA) according to the manufacturer's directions. The OD at 450 and 650 nm was determined using a microplate reader (Spectra Max M5, Molecular Devices, Inc., USA).

Serological Parameter Analysis Collected serum samples were analyzed for serological parameters using Spotchem EZ SP-4410 (Arkray, Inc., Japan) according to the manufacturer's directions.

Analysis of Hippocampal BDNF Levels The concentrations of brain-derived neurotrophin factor (BDNF) were measured in hippocampus samples using a BDNF (Mouse) ELISA Kit (KA0331, Abnova, Taiwan) in accordance with the manufacturer's directions. Briefly, hippocampal samples were removed from the − 80 °C freezer and placed on ice. A protein lysis buffer was prepared containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), sodium orthovanadate (1 mM), NaF (1 mM), 0.1% protease inhibitor cocktail (PIC; DMSO solution), and phenylmethanesulfonyl fluoride (1 mM). Lysis buffer (200 μl) was added to each sample, samples were placed on ice for 10 min, and all samples were sonicated until a homogenous mixture developed. Samples were centrifuged at 12,000*g* for 10 min, and protein-containing supernatants were removed and placed in 1.5-ml tubes. Protein levels of each sample were quantitated by Bio-Rad protein assay dye according to the manufacturer's instructions (Bio-Rad, USA). Levels of BDNF in the hippocampus were determined at wavelengths of 450 nm using a microplate reader (Spectra Max M5, Molecular Devices, Inc., USA).

Comet Assay The comet assay was performed as described previously with modifications (Pu et al. 2009). For the analysis of DNA damages in different regions of the brain, immediately after sacrifice, the brain cortex, cerebellum, and hippocampus were washed, minced in cold PBS containing 10% dimethyl sulfoxide (DMSO) and 1 mM EDTA, filtered (40-μm filter), and centrifuged (at 200×*g* for 5 min at 4 °C). The cell pellets were pooled, snap frozen in liquid nitrogen, and kept at − 80 °C. For white blood cells (WBC), blood was drawn by direct heart puncture into heparinized syringes, transferred to heparinized tubes, and centrifuged (1600*g*, 4 °C, 10 min). The white cell layer (buffy coat) was removed, pooled, snap frozen in liquid nitrogen, and kept at − 80 °C. The procedure for hepatocyte collection was previously described (Sasaki et al. 1997). Briefly, livers were removed from toluene-treated hamsters and homogenized using a Dounce homogenizer in a chilled homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA KCl solution. To obtain the nuclei, the homogenate was centrifuged at 700*g* for 10 min at 0 °C, and the precipitate was resuspended in chilled homogenizing buffer at 1 g organ weight per ml and stored at − 80 °C.

The cell pellets were resuspended in cold PBS, and a 10-μl aliquot was mixed with 70 μl 1% low-melting agarose and applied onto Trevigen CometSlides. Cells were lysed by placing the slides in a Coplin jar containing 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% sodium *N*-lauroylsarcosine, 10% DMSO, and 1% Triton X-100 (pH 10) at 4 °C for at least 1 h. For formamidopyrimidine glycosylase (FPG) digestion, slides were washed three times in Tish-HCl (0.4 M, pH = 7.5) for 10 min, and FPG (2 U/slide) was added onto the slides at 37 °C for 1 h. After that, slides were immersed in electrophoresis solution (0.3 M NaOH and 1 mM Na_2EDTA, pH > 13) for 30 min. Electrophoresis was then carried out at 1.3 V/cm for 20 min in the same solution. Slides were washed three times in Tish-HCl (0.4 M, pH = 7.5) for 10 min and in water for another 10 min. Comets were stained with ethidium bromide (2 μg/ml). Fifty nuclei per one slide per organ were examined at × 400 magnification using a fluorescence microscope equipped with a green filter and analyzed by using the Comet Imager V2.2 (MetaSystems Inc., Germany). The tail parameters used in this study to analyze toluene-induced DNA damages were the tail moment (TM) and the olive tail moment (OTM). The TM was a product of the percentage of DNA in the tail (% tail DNA) multiplied by the tail length. The OTM was defined as the percentage of DNA in the tail multiplied by the length between the center of the head and tail, which was defined by Olive et al. (2012).

Statistical Analysis Data are presented as the mean values ± SEM and were analyzed by Student's *t* test. A *p* value of < 0.05 was considered to indicate statistical significance.

Results

Acute Exposure of Toluene Induced Genotoxicity in Different Regions of the Brain

Previous studies have shown that toluene biotransformation leads to the generation of reactive oxygen species that cause oxidative stress and DNA damages (Murata et al. 1999). However, the genotoxicity of toluene in different brain regions has never been studied before. The comet assay is a rapid and sensitive tool to analyze chemically induced DNA damages, detecting strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites (Collins 2004). Here, mice were intraperitoneally injected with toluene (5 or 15 g/kg) or corn oil as a control, sacrificed after 2 h, and DNA damages in different regions of the brain, hepatocytes, and leukocytes were analyzed using the comet assay (Fig. 1a). The results in Fig. 2a–c show that acute exposure of toluene induced significant levels of DNA damages in the hippocampus, cerebellum, and cortex in a dose-dependent manner ($p < 0.05$). However, there were no significant levels of DNA damages observed in hepatocytes or leukocytes of mice following toluene exposure (Fig. 2d, e).

Previous studies have shown that in vivo exposure to various doses of toluene (0.5, 1.0, and 1.5 g/kg ip) elicited an elevation of ROS generation in different regions of the brain as well as in the blood (Mattia et al. 1993). Thus, we further used a comet assay in combination with FPG, which incises 8-hydroxy-deoxyguanosine (8-OH-dG) and the imidazole ring-opened adducts of purines. These adducts are generated in cells under oxidative stress (Tchou et al. 1994) to analyze oxidative DNA damages. Using a comet assay with FPG enzyme digestion, we found that oxidative DNA damages were significantly increased after toluene exposure in the hippocampus and cerebellum ($p < 0.05$) (Fig. 3a–c). Interestingly, a higher dose of toluene (15 g/kg) also induced DNA strand breakage in leukocytes and hepatocytes (Fig. 3d, e). These results indicate that toluene-induced DNA damages in different regions of the brain were probably due to oxidative stress following acute exposure of toluene.

Combined Effect of Toluene Exposure and Allergic Stimulation on Genotoxicity in Different Regions of Brain

Previous studies have suggested that allergic stimulation may influence the threshold for toluene sensitivity due to the modulation of neurotrophin-related genes (Win-Shwe and Fujimaki 2010). To investigate whether the allergic stimulation affects the genotoxicity of toluene in different regions of the brain, mice were immunized with OVA during the toluene exposure period (Fig. 1b, c). To monitor the allergic condition of mice, the total plasma level of anti-OVA IgG1 was

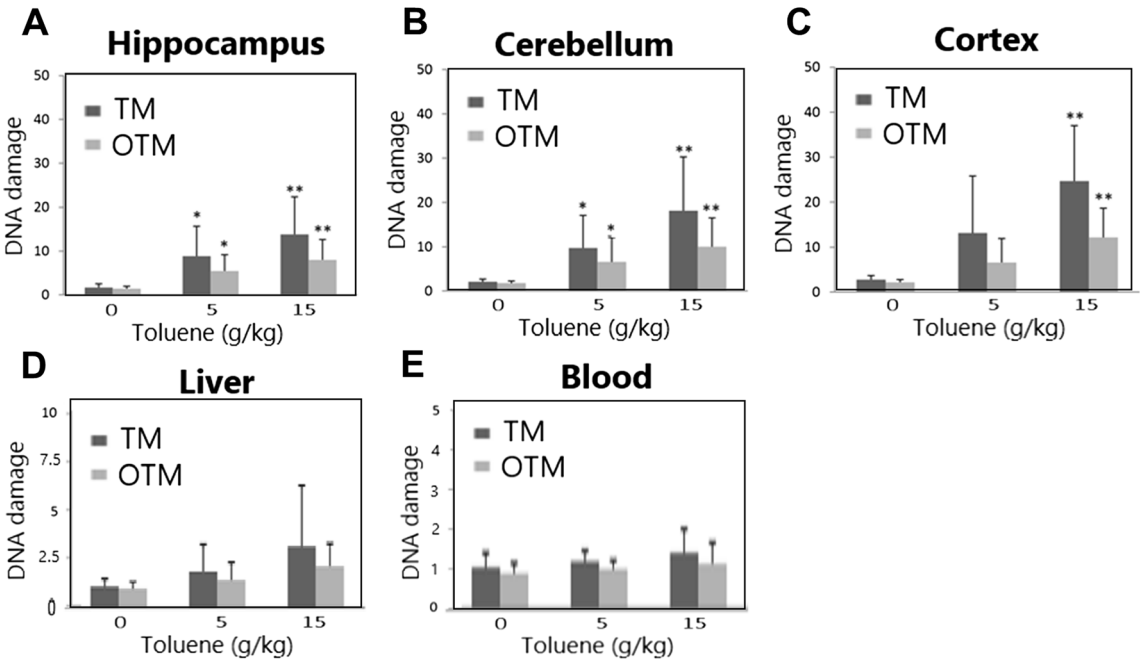

Fig. 2 Genotoxicity of acute toluene exposure in different regions of brain, hepatocytes, and leukocytes using the comet assay. Toluene-induced DNA damages were detected in the hippocampus (**a**), cerebellum (**b**), cortex (**c**), hepatocytes (**d**), and leukocytes (**e**) of animals treated with injection of toluene (5 or 15 g/kg, $n = 9$ each dose) intraperitoneally or corn oil as a control ($n = 9$) for 2 h. DNA damages were analyzed using the comet assay as described in the "Materials and Methods." TM indicates the tail moment of comet, and TOM indicates the olive tail moment of comet. Data are presented as the mean ± SD. Student's *t* tests were used to determine statistical significance, and two-tailed *p* values are shown. *$p < 0.05$ vs control group

measured, and the results showed a significant increase in IgG1 in immunized mice subjected to OVA sensitization and challenge (Supplementary Fig. 1). The results in Fig. 4 show that there was no significant difference in mouse body weight within different animal groups (Fig. 4a). In addition, the values of GOT, GPT, and BUN were not significantly different between the filtered air- and toluene-exposure groups (Fig. 4b–d). There was a significant increase in GOT and BUN but these were under the normal ranges in OVA-immunized mice compared to control mice (Fig. 4b–d). These data indicate that immunization with OVA or chronic exposure of toluene in this experimental design resulted in no serious liver or kidney toxicities. Similar to acute exposure of toluene (Figs. 2 and 3), we found that chronic exposure of toluene induced significant DNA damages in the hippocampus, cerebellum, and cortex ($p < 0.05$) but not in leukocytes in control mice (Fig. 5). However, a significant increase in DNA damages was observed in the hippocampus and leukocytes of OVA-immunized mice following toluene exposure ($p < 0.05$). Interestingly, toluene induced significantly lower DNA damages in the hippocampus and cerebellum of OVA-immunized mice compared to that of control mice ($p < 0.05$).

Because neurotrophin in the hippocampus has been shown to affect the threshold for toluene sensitivity (Win-Shwe and Fujimaki 2010; Win-Shwe et al. 2010b), we further investigated whether the expression of neurotrophin in the hippocampus affects toluene-induced genotoxicity. The results showed that a significant increase in brain-derived neurotrophic factor (BDNF) protein expression was observed in the hippocampus in the OVA-immunized mice group ($p < 0.05$) compared to the control mice group (Fig. 6). However, toluene did not induce, but slightly decreased, BDNF expression in the hippocampus in both the control and OVA-immunized mice groups.

Discussion

Toluene is one of the most widely produced and used industrial solvents worldwide as well as the most widely abused inhaled volatile substance (ATSDR 2000). The neurobehavioral and neurotoxic effects of toluene are well recognized; however, its genotoxicity is still under discussion (EPA 2005). Previous studies have shown that toluene induces ROS production during its metabolism (Murata et al. 1999). Individuals with different immunogenetic backgrounds have different sensitivities to toxic chemical exposure. It has been suggested that allergic stimulation may influence the threshold for toluene sensitivity due to the modulation of neurotrophin-related genes (Win-Shwe and Fujimaki 2010). Therefore, we hypothesized that toluene-induced genotoxicity in brain regions and allergic stimulation affected the sensitivity to toluene-induced genotoxicity. Here, our results show, using a comet assay, that exposure of toluene induced higher

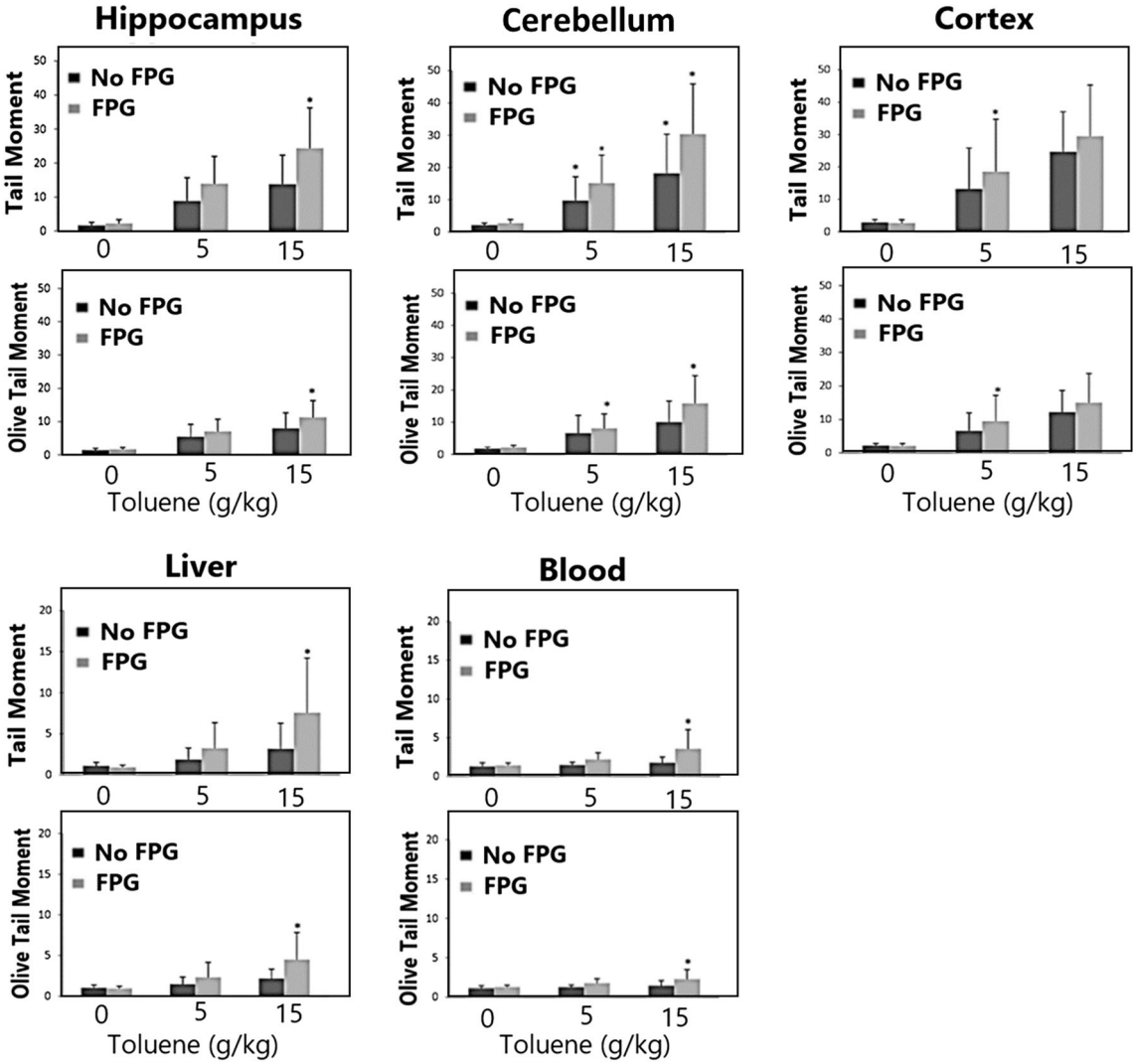

Fig. 3 Genotoxicity of acute toluene exposure in different regions of brain, hepatocytes, and leukocytes using the comet assay with formamidopyrimidine glycosylase (FPG) digestion. Toluene-induced DNA damages were detected in the hippocampus (**a**), cerebellum (**b**), cortex (**c**), hepatocytes (**d**), and leukocytes (**e**) of animals ($n = 9$) treated with injection of toluene (5 or 15 g/kg, $n = 9$ each dose) intraperitoneally or corn oil as a control ($n = 9$) for 2 h. Detection of DNA damages were performed using the comet assay with or without FPG digestion as described in the "Materials and Methods" section. TM indicates the tail moment of comet, and TOM indicates the olive tail moment of comet. Data are presented as the mean ± SD. Student's t tests were used to determine statistical significance, and two-tailed p values are shown. $^{*}p < 0.05$ vs control group; $^{\#}p < 0.05$ between groups with and without FPG digestion

genotoxicity in the hippocampus, cortex, and cerebellum in control mice compared to OVA-immunized mice. To our knowledge, this is the first report showing that toluene induced genotoxicity in the hippocampus of allergic animals, and in the hippocampus, cerebellum, and cortex of non-allergic animals, and this may contribute to the neurotoxicity of toluene.

Toluene is a well-known neurotoxicant, and the central nervous system is a primary target in both acute and chronic exposure of toluene (EPA 2005). However, the mechanisms whereby toluene exerts its effects in the brain are not fully understood. Exposure to low concentrations of toluene leads to persistent deficits in spatial learning and memory function in rat models (von Euler et al. 2000). On the other hand, a study showed that exposure to high concentrations of toluene had a significant impact on cognitive and/or psychomotor function in a mouse model (Bowen and McDonald 2009). Previous studies have shown that in vivo exposure to various doses of toluene (0.5, 1.0, and 1.5 g/kg ip) elicited an elevation of reactive oxygen species (ROS) generation in different regions of the brain, and the hippocampus had the highest induced levels of ROS (Mattia et al. 1993). DNA damages caused by ROS, including altered bases, abasic sites, and single- and double-strand breaks, are known to be a neurotoxic factor in aging and in the pathogenesis of multiple neurological disorders (Madabhushi et al. 2014; McKinnon 2013). Here, our results show that acute and chronic exposure of toluene-induced oxidative DNA damages in the

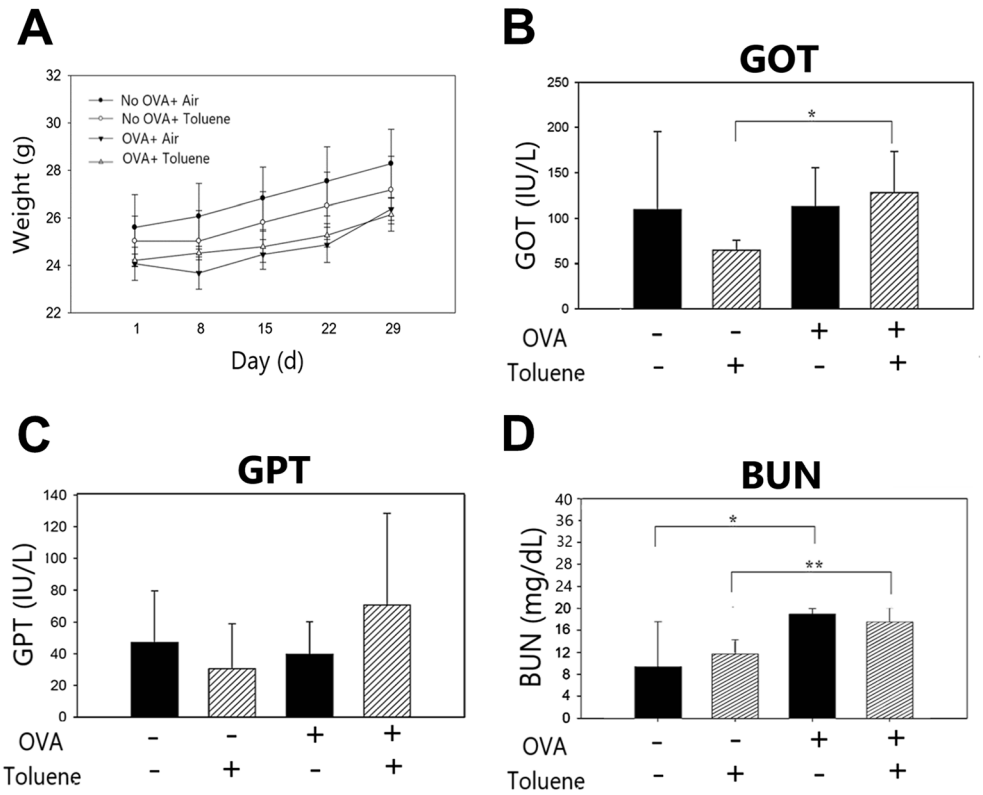

Fig. 4 Means of body weights and chemical analysis of serums obtained from mice exposed to toluene vapor. **a** Each group of mice ($n = 6$) was subjected to daily toluene exposure for 4 weeks, and body weight was measured every 7 days. Data are presented as the mean ± SD (4 weeks). **b** Each group of mice ($n = 6$) was subjected to daily toluene exposure for 4 weeks. After sacrifice, serums were collected and then analyzed for the levels of GOT, GPT, and BUN. Data are presented as the mean ± SD. Student's t tests were used to determine statistical significance, and two-tailed p values are shown. *$p < 0.05$. GOT glutamic oxaloacetic transaminase, GPT glutamic pyruvic transaminase, BUN blood urea nitrogen

hippocampus, cerebellum, and cortex may contribute to the neurotoxicity of toluene in these brain regions (Fig. 3a–c). Because oxidative DNA damages are most likely to occur in neurons, neuronal cells are equipped with a certain repair pathway, i.e., base excision repair (Jackson and Bartek 2009). Therefore, the DNA repair system for toluene-induced genotoxicity in the brain requires further investigation.

Toluene is defined as a class 3 substance (not classifiable as carcinogenic to humans) (EPA 2005; IARC 1999). Furthermore, previous results also show no significant DNA damage events in lower organisms, including bacteria, yeast, or drosophila (McGregor 1994), as well as in various animal or human studies (IARC 1999; Wetmore et al. 2008). However, some other studies have shown evidence for genotoxic effects of toluene in leukocytes from exposed subjects (Aksoy et al. 2006; Grover et al. 2003; Heuser et al. 2005; Heuser et al. 2007). In the present study, apart from toluene-induced genotoxicity in the brain, our results also show that a high dose of toluene (15 g/kg) induced oxidative DNA damages in the leukocytes of control mice (Fig. 3e). In addition, chronic exposure of toluene caused a significant increase in DNA damages in the leukocytes of OVA-immunized mice (Fig. 5d). Therefore, differences in the findings of toluene-induced genotoxicity could be associated with the kinds of exposure model used, kinds of damage caused, and/or DNA damage analysis method used. Previous results showed that genotoxic effects of toluene exposure could be detected by the comet assay but they did not affect the micronucleus frequency (Grover et al. 2003). The micronucleus assay is used for assessing DNA damages at the chromosomal level and differs from the comet assay, which can detect repairable damage (Fairbairn et al. 1995; Gunasekarana et al. 2015).

Previous data from in vivo and in vitro animal studies strongly suggests that the hippocampus is a target for toluene (Gelazonia et al. 2006; Korbo et al. 1996; Terashi et al. 1997; Win-Shwe et al. 2007). In addition, toluene exposure may cause alterations in the hippocampal functions of individuals with underlying allergy or stress-related conditions (Win-Shwe et al. 2010b). Here, we found that toluene induced DNA damages in the hippocampus of both the control and OVA-immunized mice groups (Fig. 5a). However, significantly lower DNA damages were observed in the hippocampus of OVA-immunized mice compared to those of control mice ($p < 0.05$). It has been suggested that toluene exposure affects the function of the hippocampus by modulating neurotrophin-related genes and signaling pathways and that allergic stimulation as an immune stressor may influence the threshold for sensitivity (Win-Shwe and Fujimaki 2010). Neurotrophins, such as NGF, BDNF, and the Trk family of neurotrophin receptors, are known to play a role in the survival, differentiation, and maintenance of neurons in the hippocampus as well as other brain regions (Lu et al. 2005). BDNF is one of the most widely distributed neurotrophins in the mammalian brain, and the neuroprotective function of BDNF has been

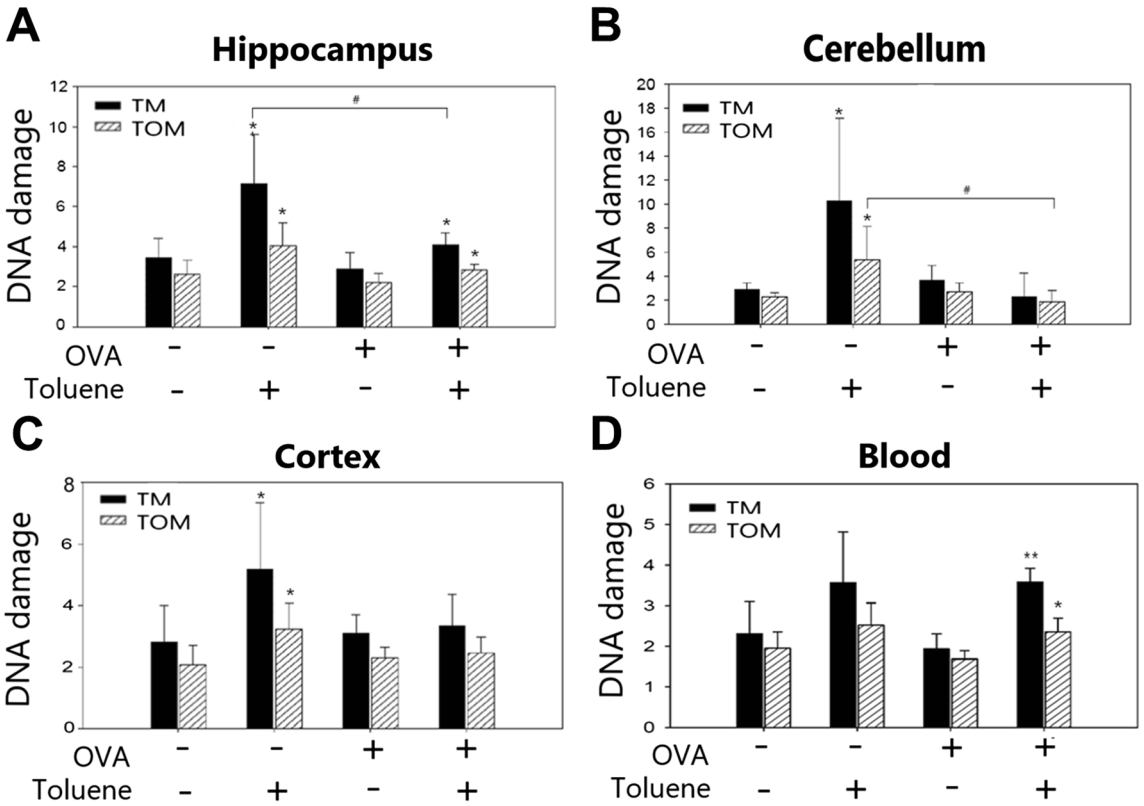

Fig. 5 Genotoxicity from chronic toluene exposure in different regions of brain, hepatocytes, and leukocytes using the comet assay. Toluene-induced DNA damages were detected in the hippocampus (**a**), cerebellum (**b**), cortex (**c**), and leukocytes (**d**) of control mice or OVA-immunized mice exposed to toluene vapor (25 ppm, $n = 6$ for each group) or filtered air as a control ($n = 6$ for each group) for 4 weeks. Immunization of mice with OVA is described in the "Materials and Methods" and Fig. 1. DNA damages were analyzed using a comet assay as described in the "Materials and Methods." TM indicates the tail moment of comet, and TOM indicates the olive tail moment of comet. Data are presented as the mean ± SD. Student's *t* tests were used to determine statistical significance, and two-tailed *p* values are shown. *$p < 0.05$ vs control group

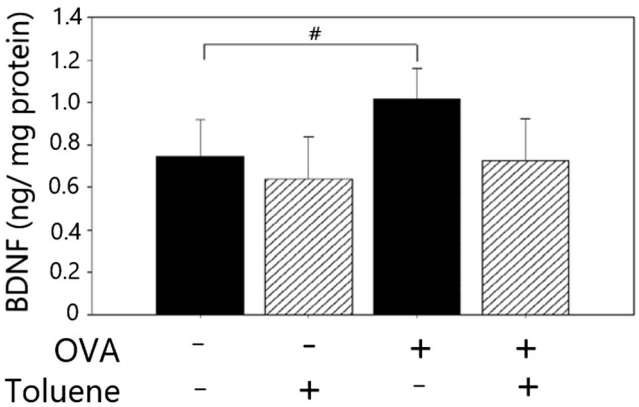

Fig. 6 Protein expression of BDNF in the hippocampus of OVA-immunized mice following toluene exposure. The expression of BDNF protein was detected in the hippocampus of control mice or OVA-immunized mice exposed to toluene vapor (25 ppm, $n = 6$ for each group) or filtered air as a control ($n = 6$ for each group) for 4 weeks. Immunization of mice with OVA is described in the "Materials and Methods" and Fig. 1. Hippocampal BDNF protein levels were analyzed using the BDNF mouse ELISA kit (Abnova) as described in the "Materials and Methods." Data are presented as the mean ± SD. Student's *t* tests were used to determine statistical significance, and two-tailed *p* values are shown. *$p < 0.05$ vs control group

widely studied (Kowianski et al. 2018; Zhao et al. 2017). In the present study, we found that BDNF protein expression was significantly increased in the hippocampus of the OVA-immunized mice group ($p < 0.05$) compared to the control mice group (Fig. 6). This is consistent with previous studies showing that increased levels of BDNF and NGF were observed in individuals with allergic diseases such as allergic rhinitis and asthma (Andiappan et al. 2011; Braun et al. 2000; Braun and Renz 2001). Furthermore, previous studies have suggested that neurons themselves produce enhanced levels of NGF and BDNF during inflammation and injury (Cho et al. 1997; Meyer et al. 1992). Increased levels of these neurotrophins, such as BDNF and NGF, have been suggested to regulate immune function as well as neuroimmune interactions (Tabakman et al. 2004; Vega et al. 2003). However, the cellular events and signal transduction pathways leading to elevated neurotrophin synthesis in inflammation remain unknown.

In addition, our results show significantly lower DNA damages in the hippocampus of OVA-immunized mice compared to that of control mice ($p < 0.05$) (Fig. 5a). Higher BDNF protein expression in OVA-immunized mice (Fig. 6)

may not have been the neuroprotective factor since there was no significant difference in hippocampal BDNF expression between control and OVA-immunized mice under toluene exposure. In contrast, our results show that toluene (25 ppm) slightly decreased hippocampal BDNF in both control and OVA-immunized mice. This result is similar to previous results showing that a high dose of toluene exposure (500 ppm) induced the expression of hippocampal BDNF, while this was not observed with a low dose (0–50 ppm) (Win-Shwe et al. 2010b). Therefore, the molecular mechanism of lower genotoxicity induced by toluene in OVA-immunized mice needs further investigation.

Taken together, the present study shows that exposure of toluene induced oxidative DNA damages resulting in genotoxicity in different brain regions, including the cortex, cerebellum, and hippocampus. Higher genotoxicity in the hippocampus induced by toluene was observed in control mice compared to OVA-immunized mice. These results provide evidence that toluene-induced genotoxicity may contribute to its neurotoxicity in different immunogenetic individuals. This study helps elucidate the molecular mechanism of neurotoxic effects caused by toluene exposure from household products, workplace settings, or inhalant abuse.

Author Contribution T.-Y.L., C.-C.C., H.-H.T., T.-Y.L., and H.-T.W. designed research; T.-Y.L., C.-C.C., H.-H.T., T.-Y.L., and H.-T.W. performed research; T.-Y.L. and H.-T.W. analyzed data; and T.-Y.L. and H.-T.W. wrote the paper.

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Compliance with Ethical Standards

The use of animals has been approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taipei, Taiwan, R.O.C. All experiments were performed in accordance with the approved guidelines.

Conflict of Interest The authors declare that they have no conflicts of interest.

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