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Toluene inhibits hippocampal neurogenesis in adult mice

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ABSTRACT

Toluene, a representative industrial solvent and abused inhalant, decreases neuronal activity *in vitro* and causes mental depression and cognitive impairment in humans. However, the effects of toluene on brain function and the sites of its action are poorly understood. This study investigated the temporal changes of neurogenesis in the hippocampus of adult C57BL/6 mice after acute administration of toluene using two immunohistochemical markers for neurogenesis, Ki-67 and doublecortin (DCX). In addition, after toluene treatment, depression-like behaviors and learning and memory tasks were examined to assess hippocampal neurogenesis-related behavioral dysfunction. The number of Ki-67- and DCX-positive cells in the dentate gyrus of adult hippocampi declined acutely between 0 h and 24 h after toluene treatment (500 mg/kg, i.p.) and increased gradually from 2 to 8 days post-administration. The level of Ki-67 and DCX immunoreactivity decreased in a dose-dependent manner within the range of toluene administered (0–1000 mg/kg). In tail suspension and forced-swim tests performed at 1 and 4 days after toluene treatment (500 mg/kg), mice showed significant depression-like behaviors compared to the vehicle-treated controls. In the contextual fear conditioning and object recognition memory test, the mice trained at 1 and 4 days after toluene treatment showed significant memory defects compared to the vehicle-treated controls. This study suggests that acute exposure to toluene reduces the rate of adult hippocampal neurogenesis and can cause hippocampal dysfunction such as depression and cognitive impairment.

1. Introduction

Hippocampal neurogenesis has been observed throughout the adult lives of mammals, and new neurons in the adult hippocampus are derived from progenitor/stem neural cells located at the border between the hilus and granular cell layer of the dentate gyrus (DG), a region called the subgranular zone (SGZ) (Kempermann et al., 1997). The rate of hippocampal neurogenesis can be changed by various factors such as age (Kuhn et al., 1996), genetic influence (Kim et al., 2009), excitatory input (Parent et al., 1997; Segi-Nishida et al., 2008), ionizing radiation exposure (Tada et al., 2000; Kim et al., 2008), physiological stimuli (Kronenberg et al., 2003), and environmental conditions (Scott et al., 1998; Young et al., 1999). Furthermore, a decline in neurogenesis in the adult hippocampus may result in neurobehavioral dysfunctions such as depression and cognitive impairment (Sahay and Hen, 2007).

Toluene is an organic solvent that is found in commercially available products including paint, adhesives, and various industrial solvents. Painters, who are frequently exposed to volatile solvents including toluene, often complain of dizziness, depression, cognitive impairment, and fatigue (Kishi et al., 1993; Sugiyama-Oishi et al., 2000; Lee et al., 2003). Furthermore, brain magnetic resonance imaging has revealed cerebral and hippocampal atrophy in toluene/solvent abusers (Kamran and Bakshi, 1998; Deleu and Hanssens, 2000). Despite the substantial epidemiological data regarding the neurobehavioral and neurotoxic effects of toluene, the sites and mechanisms of toluene action in the brain are poorly understood.

Toluene can cross readily through the blood-brain barrier (BBB) and produce central nervous system (CNS) effects similar to those of other sedative-hypnotics (Balster, 1998). In animal experiments, toluene exposure leads to changes in neurobehavioral and neurobiological functions (Wood and Colotla, 1990; Kondo et al., 1995; Riegel and French, 1999; von Euler et al., 2000; Wiaderna and Tomas, 2000; Berenguer et al., 2003). Moreover, prenatal toluene exposure results in abnormal neuronal proliferation and migration in the rat somatosensory cortex (Gospe and Zhou, 2000). However, little is known about the detrimental effects of toluene exposure on the adult hippocampus that relate to neurobehavioral functions.

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This study examined neurogenesis in the DGs of hippocampi from adult C57BL/6 mice after the acute administration of toluene using two immunohistochemical markers of neurogenesis, Ki-67 (a proliferating cell marker) (Scholzen and Gerdes, 2000) and doublecortin (DCX; an immature progenitor cell marker) (Francis et al., 1999) to elucidate the effect of toluene on adult hippocampal neurogenesis. In addition, tests evaluating depression-like behaviors, the tail suspension test (TST) and forced-swim test (FST), and learning and memory tasks, the contextual fear conditioning and object recognition memory test were performed after toluene injection to evaluate hippocampal neurogenesis-related behavioral dysfunction in adult mice exposed to toluene.

2. Method

2.1. Animals

In total, 240 male C57BL/6 mice aged 8–9 weeks (Orient Bio, Gyunggi-do, Korea) were used in this experiment. All animal experiments followed a protocol approved by the Committee for Animal Experimentation at Chonnam National University.

2.2. Toluene administration and tissue sampling

Toluene (HPLC grade, 99.8%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Toluene-treated mice were injected intraperitoneally with toluene dissolved in corn oil. Control mice were injected with only corn oil. Intraperitoneal (i.p.) injection of toluene has been reported to produce the same behavioral symptoms as inhalation (Kondo et al., 1995; Riegel and French, 1999) and is easy to handle; thus, this treatment protocol was performed in this study.

In addition, in previous studies with a rat model, a dose of 520 mg/kg toluene simulated the blood toluene levels obtained after an inhalation exposure to 3290 ppm (corresponding to ~36 mM) (Gospe et al., 1994). Also, no effect on body weight gain was observed in rats exposed to 500 mg/kg (Chen et al., 2005), and this dose of toluene exposure from postnatal days 4 to 9 enhanced the susceptibility to neurobehaviors significantly (Lee et al., 2005). Riegel et al. (2003) used 600 mg/kg (i.p.) of toluene, in the absence of an interfering ataxia that begins to be expressed at higher doses. Therefore, we used a dose of 500 mg/kg toluene to examine the behavioral changes of mice in this study.

The time-dependent effect of toluene on neural apoptosis and neurogenesis in the adult mouse hippocampus was observed at 0, 12, and 24 h, as well as at 2, 4, and 8 days ($n=4$ mice per group) after either corn oil alone (vehicle) or toluene administration (500 mg/kg, i.p.). To observe the dose-dependent effects of toluene on neurogenesis in adult hippocampi, mice were administered 0 (vehicle-treated control), 100, 500, or 1000 mg/kg of toluene. The mice were killed 24 h later and the hippocampi from each group were dissected ($n=4$ mice per group). The brain samples were processed for embedding in paraffin wax after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS).

To observe the effect of toluene on locomotor activity, depression-like behaviors, and learning and memory tasks mice were administered either corn oil alone (vehicle-treated control) or toluene in corn oil (500 mg/kg, i.p.). Each behavior test was performed at 1 and 4 days after vehicle or toluene administration.

2.3. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

DNA fragmentation was detected using *in situ* nick end labeling (TUNEL), which was performed according to the manufacturer's instructions (ApopTag® In Situ Apoptosis Detection Kit; Intergen, Purchase, NY, USA).

2.4. Immunohistochemistry

Coronal sections (5 μm thick) were cut using a microtome and deparaffinized by routine protocols before being exposed to citrate buffer (0.01 M, pH 6.0) and heated in an autoclave for 10 min. All subsequent steps were performed at room temperature. The sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes in PBS, the sections were blocked with 10% normal goat serum (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 h and then incubated for 2 h with immunohistochemical markers for proliferating cells and immature progenitor cells, i.e., monoclonal rabbit anti-Ki-67 antibody (DRM004; diluted 1:400, Acris Antibodies GmbH, Hiddenhausen, Germany) or polyclonal rabbit anti-DCX antibody (diluted 1:200, Cell Signaling Technology, Beverly, MA, USA). After three washes in PBS, the sections were reacted with biotinylated goat anti-rabbit IgG (Vector Laboratories; diluted 1:100) for 45 min. After three washes in PBS, the sections were incubated for 45 min with the avidin–biotin peroxidase complex (Vector ABC Elite Kit) prepared according to the manufacturer's instructions. After three washes in PBS, the peroxidase reaction was developed for 3 min using a diaminobenzidine substrate (DAB kit, SK-4100; Vector Laboratories) prepared according to the manufacturer's instructions. As a control, the primary antibodies were omitted for a few test sections in each experiment. After the completion of color development, the sections were counterstained with Harris's hematoxylin for 5 s, washed in running tap water for 20 min, dehydrated through a graded ethanol series, cleared with xylene, and mounted with Canada balsam (Sigma-Aldrich).

2.5. Determination of cell number

The number of cells showing the specific characteristics of proliferating cells (immunopositive for Ki-67) and immature progenitor neurons (immunopositive for DCX) in the hippocampus was scored using a histomorphometric approach (Kim et al., 2008, 2009). The observer was blind to the identity of the sample. The brain from each mouse was sampled at a level approximately 2.12 mm caudal to the bregma. For each mouse, a standardized counting area was composed of DG regions in three 5-μm-thick coronal sections chosen from a one-in-ten series of sections representing the rostral/mid-hippocampus. One coronal section was chosen from each of the three regions of the hippocampus (about 50 μm apart) such that the sections were nonoverlapping. All positively immunolabeled cells within the subgranular zone of the supra- and infra-pyramidal blades of the DG were counted. The number of immunopositive cells was averaged across the DG region in each of the three brain sections such that the mean number of immunopositive cells in the three sections of each mouse was taken as $n=1$.

2.6. Behavioral testing

2.6.1. Open-field test

Open-field analysis was used to measure the activity of the mice in a novel environment. Parameters including total moving distance (cm), ambulatory movement time (s), and ambulatory movement episodes were determined over a 5-min period using the TruScan Photo Beam Activity System (Coulbourn Instruments, Whitehall, PA, USA).

2.6.2. TST

The TST was similar to that described by Steru et al. (1985). Briefly, mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. Immobility was measured for 6 min. Immobility was defined as the absence of any limb or body movements, except those caused by respiration.

2.6.3. FST

The FST was similar to that described by Porsolt et al. (1977). Briefly, mice were gently placed in a clear plastic cylinder with a diameter of 13 cm and a height of 23 cm that was filled with 10 cm of clear water at 23–25 °C. The test duration was 6 min, and immobility was measured during the last 4 min. Immobility was defined as the absence of any horizontal or vertical movement in the water, but excluded minor movements required for the mouse to keep its head above the surface. The water was replaced before each animal began the test.

2.6.4. Contextual fear conditioning

During training, mice were placed in the operant chamber with a metal grid floor for 30 s (TruScan chamber with photobeam sensors; Coulbourn Instruments), after which a mild electric foot-shock was delivered (0.3 mA for 1 s). At 24 h after training, mice were tested and scored for the percentage of freezing. The freezing behavior, defined by immobility except for breathing, was scored once every 2 s for 2 min.

2.6.5. Object recognition memory test

Eight-week-old mice were first habituated in the training/testing chamber (42 cm L, 28 cm W, 20 cm H) for 24 h. The objects for recognition to be discriminated, which were made from plastic, had three different shapes: cubes, pyramids, and cylinders that were 3.5 cm high and could not be displaced by the mice. The chamber area and objects were cleaned with 75% ethanol between trials to prevent the buildup of olfactory cues. During training, two objects selected randomly with different shapes were presented to each mouse for 15 min. At 24 h after training, another set of objects (one old object and one novel object) was presented to the trained mice. For example, if the cube- and pyramid-shaped objects were presented during training, the cylinder-shaped object was used as a novel object during testing. The interaction of the mouse with each object, including approaches and sniffing, was scored. If the mouse had memory retention for an old object, it would show preference to the novel object during testing. The percentage preference was defined as the "number of interactions for a specific object" divided by the "total number of interactions for both objects."

2.7. Statistical analysis

The data are reported as the mean ± SE and were analyzed using a one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test for multiple comparisons. In all cases, a p value <0.05 was considered significant.

3. Results

First, we examined the histological change in hippocampal structure using hematoxylin and eosin (H&E) staining. H&E staining revealed no unusual hippocampal structure in adult mice at 6 h to 8 days after acute toluene exposure (Fig. 1A and B). Also, TUNEL-positive apoptotic nuclei were rarely detected in the CA1, CA3, and DG regions of the hippocampus after exposure to toluene (Fig. 1C and D). This suggests that acute toluene exposure does not induce neural apoptosis in the hippocampus of adult mice.

To elucidate the effect of toluene on adult hippocampal neurogenesis, two immunohistochemical markers of neurogenesis, Ki-67 (a proliferating cell marker) and DCX (an immature progenitor neuronal marker), were examined in the DGs of hippocampi from adult mice after toluene injection. Ki-67 immunoreactivity was apparent in the nuclei of cells located at the border between the granular cell layer (GCL) and the hilus of the DG, a region called the SGZ, in the adult mouse hippocampus (Fig. 2, arrows). The number of Ki-67-immunopositive cells decreased sharply between 0 [17.88 ± 0.97 nuclei/DG ($n=4$)] and 24 h after toluene injection [24 h: 9.38 ± 0.75 nuclei/DG ($n=4$, $p<0.001$ *vs.* vehicle-treated control)], whereas the number of Ki-67-positive cells gradually increased from 2 to 8 days after toluene injection (Fig. 2E). DCX immunoreactivity was observed in the cytoplasm of cells located in the GCL adjacent to the hilus of the DG in the hippocampus of adult mice (Fig. 3). The immunoreaction revealed the ramified body shape of the cells in the GCL. To quantify DCX-labeling, cells were counted as DCX-immunopositive if the cell had both immunopositively labeled cytoplasm and nuclei counterstained with hematoxylin (Fig. 3, arrows). The number of DCX-immunopositive cells declined between 0 [50.25 ± 4.82 nuclei/DG ($n=4$)] and 12 h after toluene injection [36 ± 4.06 nuclei/DG ($n=4$, $p<0.05$ *vs.* vehicle-treated control)], reaching the lowest number of DCX-positive cells at 24 h [32.25 ± 4.07 nuclei/DG ($n=4$, $p<0.05$ *vs.* vehicle-treated control)]. Then, the number of labeled cells increased gradually from 2 to 8 days after toluene injection (Fig. 3E).

As shown in Fig. 4A, Ki-67 immunoreactivity in the SGZ of the DG decreased progressively with increasing dose of injected toluene (0–1000 mg/kg). The number of Ki-67-positive proliferating cells declined sharply at 0 to 100 mg/kg toluene, but leveled off slowly as the dose was increased. A decrease in the number of DCX-positive cells in the DG was also observed with increasing administration dose. A significant decrease was observed in the number of DCX-positive cells between 0 and 500 mg/kg, and the change leveled off at 500–1000 mg/kg (Fig. 4B).

To evaluate hippocampal neurogenesis-related behavioral dysfunction in adult mice exposed to toluene, basal locomotor activity, depression-like behaviors, and learning and memory tasks were performed in adult mice at 1 and 4 days after toluene (500 mg/kg) injection.

The basal locomotor activity of vehicle-treated control mice and toluene-treated mice at 1 and 4 days after injection was examined by placing mice in a novel environment and performing open-field analysis. Open-field analysis quantified overall activity that can affect the motivation and performance of the mice. The vehicle-treated controls and the toluene-treated mice showed comparable moving distances and ambulatory movement times and episodes at 1 and 4 days after injection (see Table 1). This suggests that toluene exposure does not alter locomotor activity significantly in adult mice.

TST and FST have been recognized as useful experimental paradigms for assessing depression-like behavior and the activity of antidepressants. These tests were performed at 1 and 4 days after toluene injection. Toluene-treated mice exhibited a longer duration of immobility during the TST compared to vehicle-treated mice at 1 (vehicle-treated control: 93 ± 5.1 s, toluene-treated mice: 139.6 ± 18.4 s; $n=7$, $p<0.05$) and 4 days (vehicle-treated control: 108.1 ± 3.8 s, toluene-treated mice: 195.6 ± 6.1 s; $n=7$, $p<0.001$) after injection (Fig. 5A). Similarly, immobility time in the FST increased significantly in toluene-treated mice as compared to the vehicle-treated control group at 1 (vehicle-treated control: 167 ± 6.5 s, toluene-treated mice: 206.6 ± 3.3 s; $n=7$, $p<0.05$) and 4 days (vehicle-treated control: 108 ± 7.1 s, toluene-treated mice: 215.3 = 2.8 s; $n=7$, $p<0.05$) after injection (Fig. 5B). Conversely, climbing time [1 day after toluene injection: 10 ± 0.3 s, $p<0.001$ *vs.* vehicle-treated controls (12.7 ± 0.4 s); 4 days after toluene injection: 8.9 ± 0.3 s, $p<0.01$ *vs.* vehicle-treated controls (11.9 ± 0.7 s)] and swimming time [1 day after toluene injection: 81.9 ± 5.7 s, $p<0.01$ *vs.* vehicle-treated controls (116.4 ± 6.9 s); 4 days after toluene injection: 81.9 = 2.2 s, $p<0.001$ *vs.* vehicle-treated controls (121.1 = 8.7 s)] measured during the FST were both significantly lower in toluene-treated mice than in the vehicle-treated control group (Fig. 5B).

Mice were examined by contextual fear conditioning ($n=8$ per group), which is a hippocampus-related learning paradigm. During training, both vehicle- and toluene-treated mice displayed minimal freezing (Fig. 6A). Their sensitivity to electric foot-shock was further

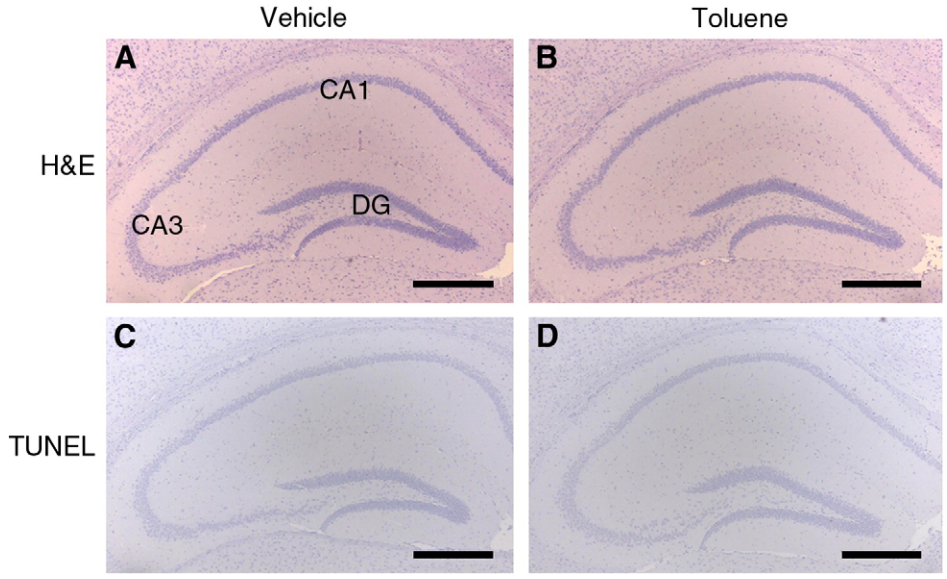

Fig. 1. Histological results for the vehicle-treated controls 24 h after injection with only corn oil (A and C) and toluene-treated mouse hippocampus at 24 h after administration of 500 mg/kg toluene (B and D). No unusual hippocampal structure was observed in vehicle-treated controls (A) and toluene-treated mice (B). TUNEL-positive apoptotic nuclei were rarely detected in the CA1, CA3, and DG regions of the hippocampus with vehicle (C) and toluene treatment (D). Scale bars, 400 μm.

tested, and no significant differences were observed in the threshold current to elicit stereotypic responses, including flinch, vocalization, and jump/vocalization, between vehicle-treated and toluene-treated mice (data not shown). This suggests that the mice had comparable sensitivity to the electric foot-shock. The memory retention trials (testing) were carried out at 24 h after the acquisition trial (training). Vehicle-

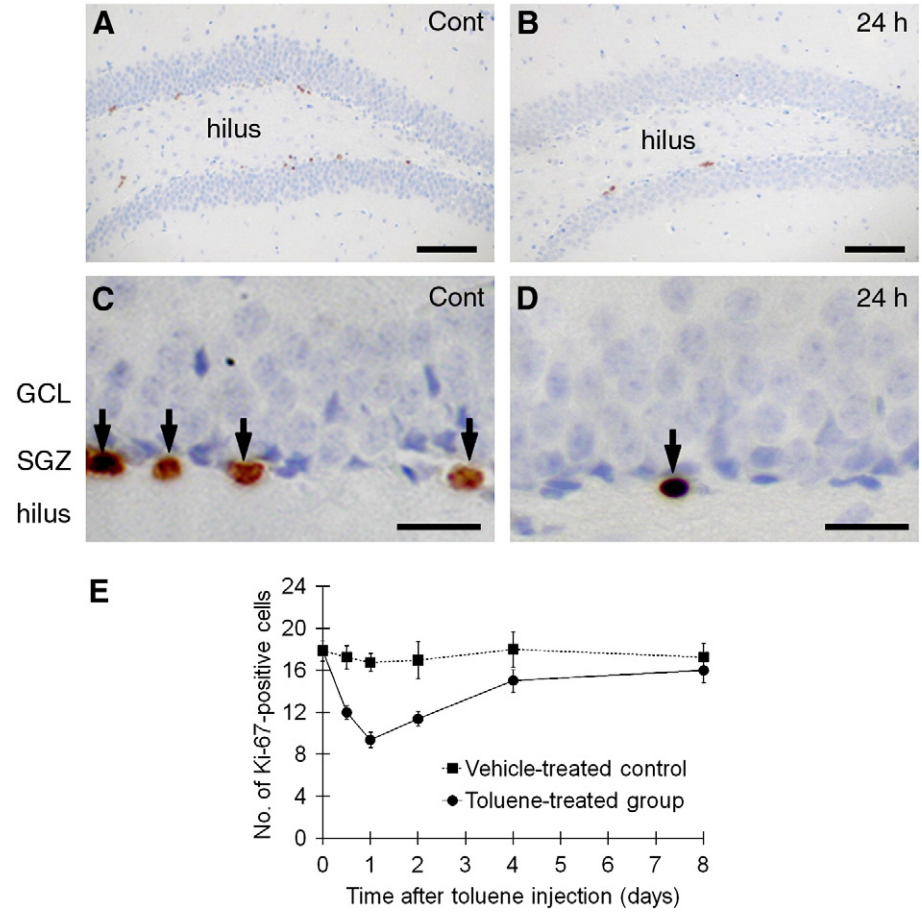

Fig. 2. Temporal profile of Ki-67 expression in the DG of the adult hippocampus after toluene treatment. (A–D) Representative images showing Ki-67-positive cells in the DGs of adult hippocampi in control mice (vehicle only) and toluene-treated mice (500 mg/kg) 24 h after injection. (E) The number of Ki-67-positive cells in the DG markedly decreased 12–24 h after toluene injection and gradually increased at 8 days after toluene injection. The cells were counterstained with hematoxylin (A–D). GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars, 20 μm (C and D), 100 μm (A and B). The data are reported as the mean ± SE (for E, $n = 4$ per group).

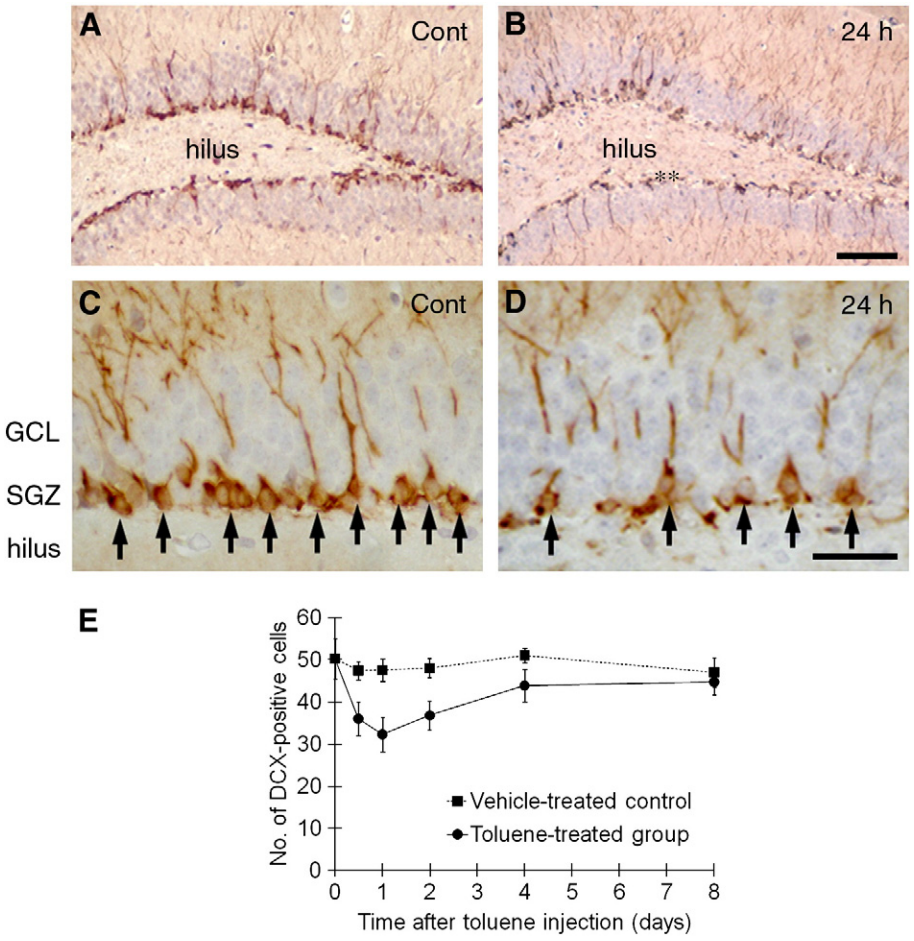

Fig. 3. Temporal profile of DCX expression in the DG of the adult hippocampus after toluene treatment. (A–D) Representative images showing the DCX-positive cells in the DGs of adult hippocampi taken from the controls (24 h after vehicle injection) and mice 24 h after toluene injection (500 mg/kg). (E) The number of DCX-positive cells in the DG decreased markedly at 12–24 h after toluene injection and then increased gradually at 8 days after toluene injection. The cells were counterstained with hematoxylin (A–D). GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars, 20 μm (C and D), 100 μm (A and B). The data are reported as the mean ± SE (for E, $n = 4$ per group).

treated mice showed a significant increase in freezing (mean ± SE%) when tested 24 h after training (1 day after injection, 69.9 ± 7.9%; 4 days after injection, 71.6 ± 3.9%; Fig. 6A). The mice trained at 1 and 4 days after toluene injection showed significantly lower freezing during the test (1 day after injection, 42.8 ± 9.6%, $p < 0.05$ *vs.* vehicle-treated control; 4 days after injection, 41.04 ± 8%, $p < 0.01$ *vs.* vehicle-treated control; Fig. 6A).

We further examined mice ($n = 5$ per group) by a sensitive hippocampus-dependent paradigm, object recognition memory test (Kim et al., 2008). Vehicle- and toluene-treated mice at 1 and 4 days after toluene injection displayed an equal preference for the two objects during training. No significant difference was seen in the total time spent exploring both objects during the training trial between the groups (data not shown). During the test, the preferences

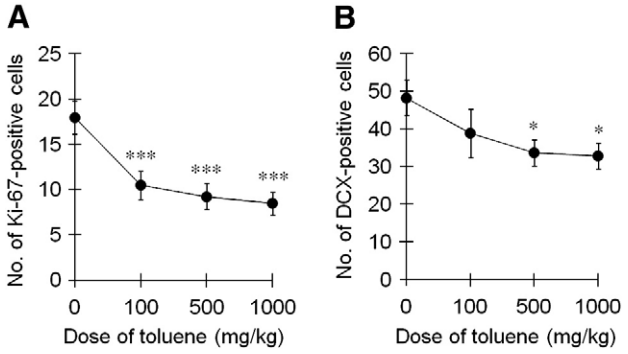

Fig. 4. Dose-dependent changes in Ki-67 and DCX immunoreactivity in the DGs of adult mouse hippocampi at 24 h after toluene treatment. (A) The number of Ki-67-positve cells in the DGs. (B) The number of DCX-positive cells in the DGs. Ki-67 and DCX immunoreactivity in the SGZ of the DG decreased as the dose of toluene treatment increased (0–1000 mg/kg). The data are reported as the mean ± SE ($n = 4$ per group). $^{*}p < 0.05$ *vs.* vehicle-treated controls (24 h after only corn oil injection), $^{***}p < 0.001$ *vs.* vehicle-treated controls (24 h after only corn oil injection).

Table 1
Open-field analysis of mice placed in a novel environment at 1 and 4 days after acute toluene administration.

	Distance (cm)	Movement time (s)	Movement episodes
(1 day after injection)			
Vehicle-treated control	475.32 ± 19.14	219.33 ± 4.72	36.33 ± 2.28
Toluene-treated group	424.98 ± 40.28	202.13 ± 10.93	33.38 ± 3.53
(4 days after injection)			
Vehicle-treated control	438.37 ± 57.02	209.83 ± 14.27	32.33 ± 3.23
Toluene-treated group	500.95 ± 33.08	227.88 ± 6.54	28.13 ± 1.14

Open-field data for vehicle-treated controls (injected with corn oil, i.p., $n = 6$) and toluene-treated mice (injected with toluene in corn oil at 500 mg/kg, i.p., $n = 8$) at 1 and 4 days after injection. No significant differences were found in movement distance ($p = 0.332$, 1 day after injection; $p = 0.334$, 4 days after injection), ambulatory movement time ($p = 0.223$, 1 day after injection; $p = 0.233$, 4 days after injection), or the number of ambulatory movement episodes ($p = 0.528$, 1 day after injection; $p = 0.195$, 4 days after injection) between vehicle-treated controls and toluene-treated mice at 1 and 4 days after injection. The data are reported as the mean ± SE.

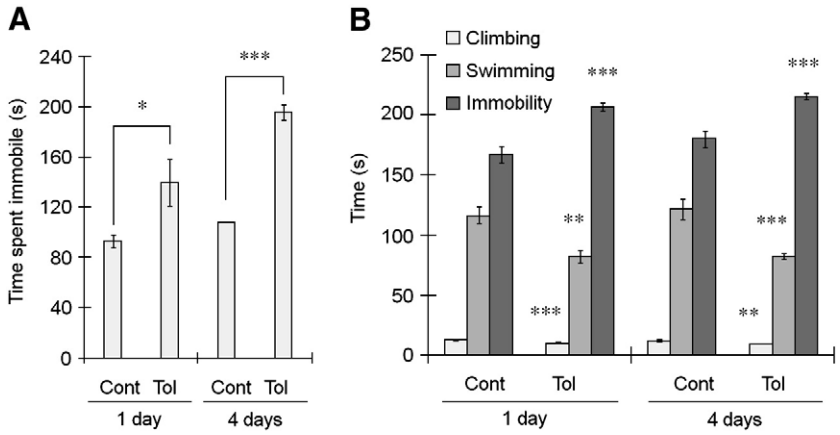

Fig. 5. The effect of toluene injection on immobility measured during the TST and FST in adult mice. Vehicle (corn oil) or toluene (500 mg/kg) was injected at 1 day and 4 days before the test. In the TST, the time spent immobile was significantly higher in toluene-treated mice than in vehicle-treated control mice (A). In the FST, the time spent climbing and swimming was significantly lower, and the time spent immobile was significantly higher in the toluene-treated mice compared with the vehicle-treated control mice (B). The values reported are the mean ± SE ($n = 7$ per group). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* vehicle-treated controls.

(mean ± SE%) toward a novel object were 80.5 ± 4.5% and 75.7 ± 1.2% in the mice trained at 1 and 4 days after vehicle injection, respectively, and 55.8 ± 3.7% and 57.36 ± 2.1% in the mice trained at 1 and 4 days after toluene injection, respectively (Fig. 6B). Thus, toluene-treated mice showed memory deficits (1 day after injection, $p<0.01$ *vs.* vehicle-treated controls; 4 days after injection, $p \pm 0.01$ *vs.* vehicle-treated controls) in object recognition memory.

4. Discussion

This study demonstrates that the acute administration of toluene reduces the number of Ki-67 (proliferating cells)- and DCX-positive cells (immature progenitor neurons) in the adult mouse hippocampus and induces depression-like behaviors in the TST and FST, and cognitive impairments in the contextual fear conditioning and object recognition test. This indicates that toluene exposure interferes with adult hippocampal neurogenesis and related functions.

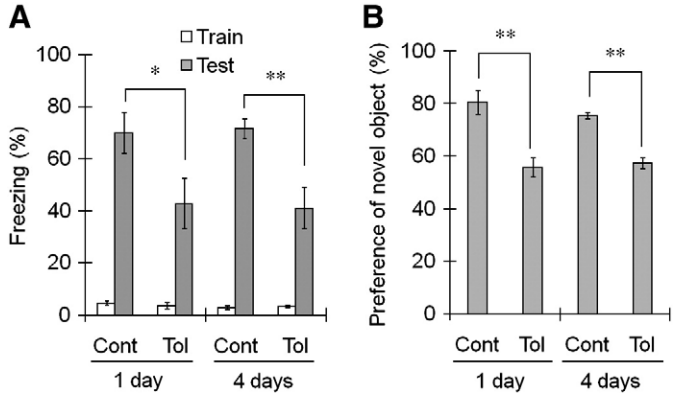

Fig. 6. Mice with acute toluene treatment exhibited cognitive deficits in contextual fear conditioning and object recognition memory test. (A) Vehicle-treated controls (1 day and 4 days after injection of only corn oil) and toluene-treated mice (1 day and 4 days after 500 mg/kg toluene treatment) were trained by contextual fear conditioning ($n = 8$ per group). The trained mice were tested 24 h after training. The freezing was recorded. Mice trained at 1 day and 4 days after toluene treatment exhibited weaker learning and memory formation than the vehicle-treated controls, as indicated by the significantly lower freezing on testing. (B) Vehicle-treated controls (1 day and 4 days after injection of only corn oil) and toluene-treated mice (1 day and 4 days after 500 mg/kg toluene treatment) were examined ($n = 5$ per group) for object recognition memory. During training, two objects were presented to each mouse for 15 min. After 24 h, one of the old objects was replaced with a novel object (testing). If the mouse remembered the old object, it would spend more time with the novel object during testing, as indicated by the higher percentage of object preference. During testing, a significant difference was observed in the novel object preference between the vehicle-treated controls and toluene-treated mice during testing. The data are reported as the mean ± SE. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* vehicle-treated controls.

Occupational and environmental exposure to toluene is frequently encountered. Several clinical and epidemiological studies showed that long-term exposure to toluene may result in chronic toxic encephalopathy (Echeverria et al., 1991; Kishi et al., 1993; Balster, 1998; Kamran and Bakshi, 1998; Deleu and Hanssens, 2000; Wiaderna and Tomas, 2000; Lee et al., 2003). In this study, acute toluene exposure inhibited hippocampal neurogenesis in adult mice without significant induction of apoptosis. Gospe and Zhou (2000) reported that prenatal toluene exposure results in abnormal neuronal proliferation and migration. A recent *in vitro* study demonstrated that toluene disrupts synapse formation and maintenance during neural development, which would be expected to generate a significant impact on the subsequent progression of neurogenesis (Lin et al., 2009). However, these authors did not detect considerable neuronal injury via the assessment of general morphology and colorimetric viability assay. Moreover, toluene inhibits the muscarinic receptor-mediated cytosolic Ca^{2+} response in neural precursor cells (Ma et al., 2002), suggesting that toluene can disrupt neural cell development because cytosolic Ca^{2+} and muscarinic receptors play important roles in cell proliferation and differentiation during neural precursor cell development (Zhou et al., 2004). Therefore, it would be interesting in the future to inspect the effect of toluene on the cytosolic Ca^{2+} response and related signaling molecules, crucial for neurogenesis, in the adult mouse hippocampus.

In clinical cases, workers with chronic toluene exposure display symptoms such as insomnia, dizziness with headache, memory impairment, and depression (Kishi et al., 1993; Sugiyama-Oishi et al., 2000; Lee et al., 2003). Mental depression and cognitive impairment may be due not only to the changes in neurotransmitter concentrations and receptor activity levels, but also to impairment of brain plasticity, and tissue remodeling and alterations in adult hippocampal neurogenesis (Cameron et al., 1998; Duman et al., 1999; Gould and Tanapat, 1999; Jacobs et al., 2000; Aonurm-Helm et al., 2008). Therefore, the reduction in hippocampal neurogenesis in adults by toluene exposure may induce depression. We additionally examined the deleterious effect of toluene exposure on hippocampal function through behavioral evaluations for depression and learning and memory. The results showed remarkable depression-like behaviors in the TST and FST, and cognitive impairments in the contextual fear conditioning and object recognition memory test without significant alteration of locomotor activity.

In addition, toluene is well-known to act as a noncompetitive glutamatergic NMDA receptor antagonist in different experimental

preparations (Cruz et al., 1998, 2003; Bale et al., 2005). However, opposing results have been reported recently, whereby the mRNA expression of NR2B increased significantly in the hippocampus of mice chronically exposed to 50 ppm of toluene (Ahmed et al., 2007) and toluene exposure during synaptogenesis specifically increased the levels of NR2A subunits in the hippocampus and cerebellum on postnatal day 30 (Lee et al., 2005). Since NR2A was the main subunit affected, the alterations in NR2A protein levels may play an important role in the manifestation of neurotoxicity induced by toluene exposure in hippocampus-related depression. However, further studies are needed to clarify the precise mechanism of toluene-induced depression-like behavior and memory impairment, and to establish a definite relationship between the alterations in hippocampal neurogenesis and behavior. Furthermore, because other regions were not included in this analysis, the possibility remains that neural changes in other regions may contribute to the toluene-induced depression-like behavior and cognitive impairment observed in this study.

In summary, the number of proliferating and migrating cells in the DGs of hippocampi in adult C57BL/6 mice was significantly reduced after toluene exposure. The lower rate of hippocampal neurogenesis may be causally related to the depression-like behavior and the cognitive impairment also observed after toluene exposure in adult mice.

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