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Neurotoxicity of methotrexate to hippocampal cells *in vivo* and *in vitro*

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ABSTRACT

This study investigated whether methotrexate (MTX) is neurotoxic to neural progenitor cells in the hippocampus of adult mice and whether it affects hippocampus-dependent behaviors. In addition, the cytotoxicity of MTX was elucidated in rat immature and mature hippocampal cultured cells. The number of Ki-67 (proliferating cell marker)- and doublecortin (immature progenitor neuron marker)-positive cells were significantly time- and dose-dependently changed in the dentate gyrus of adult hippocampi after MTX treatment. A learning and memory task (object recognition memory test) and depression-like behavior test (tail-suspension test) were performed after MTX treatment to assess hippocampal neurogenesis-related behavioral dysfunction. MTX-treated mice showed significant depression-like behaviors and memory defects. The cytotoxicity of MTX in immature hippocampal cells varied in a dose-dependent pattern, but was not changed in the mature cells. MTX induced marked apoptotic changes in immature hippocampal cells, with increase in active caspase-3 and cleaved poly (ADP-ribose) polymerase expressions. Results of this study suggest that the neurotoxic effect of MTX inhibits the proliferation of hippocampal progenitor cells and can cause hippocampal dysfunction, such as depression and cognitive impairment. Additionally, the significantly greater caspase-dependent MTX sensitivity of immature hippocampal cells suggests that the susceptibility of such hippocampal cells depends on their maturation.

1. Introduction

Cytostatic chemotherapy is often applied in modern oncology; cancer patients live longer and even survive. However, there is increasing clinical interest in its negative side effects on other systems, including cardiological (*i.e.* arrhythmia) [1], gastrointestinal (*i.e.* vomiting and mucositis) [2], and psychological disorders (*i.e.* depression and cognitive impairment) [3–5].

Methotrexate (MTX) is used in the treatment of many diverse malignancies and in the therapy of diverse autoimmune diseases, such as rheumatoid arthritis and psoriasis, due to its anti-inflammatory and immunosuppressive effects [6,7]. MTX can be safely administered over a wide dose range in maintenance chemotherapy for acute lymphoblastic leukemia [8]. It exerts antineoplastic effects by competitively inhibiting folate-dependent biochemical processes, thus inhibiting DNA synthesis [9]. However, MTX is a frequently used cytotoxic agent in the clinic and is associated with acute and chronic neurotoxicity [10].

Neurotoxicity is a common and often dose-limiting complication of chemotherapy treatment [11]. The incidence markedly increases when the blood–brain barrier is either overwhelmed or bypassed [12]. Most common neurological complications involve acute alterations in consciousness, seizures, cerebral infarctions, paralysis, neuropathies, leukoencephalopathy, and ototoxicity [13]. Several studies have reported that various chemotherapy-induced neurotoxicities are related to various mechanisms. Anticancer drugs have neurotoxic properties *in vitro* and *in vivo*, especially in mature neurons [14]. Excitotoxic mechanisms and caspase-mediated cell death contribute to the neurotoxicity of these compounds *in vitro* in neuronal cultures and *in vivo* in the developing rat brain [14]. Additionally, neurotoxicity of mature neuronal cells exposed to anticancer drugs can be alleviated by inhibiting the Rho signaling pathway [15]. Nevertheless, little is known about the precise mechanism of the toxicological effect of chemotherapeutic agents on brain function, especially related to neuronal survival/death.

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Adjuvant chemotherapy is frequently applied in the treatment of cancer. The survival rate of patients treated with cytostatics is high; however, the treatment is associated with both short- and long-term side effects. One of the potential long-term effects is cognitive impairment, which primarily manifests as a decrease in attention/concentration, speed of information processing, and memory [16]. For example, some breast cancer survivors experience cognitive defects following chemotherapy [17–19]. More patients treated with high-dose chemotherapy than patients treated with standard-dose chemotherapy show a defect in cognitive performance compared with healthy control subjects [20,21]. In addition, several *in vivo* studies have suggested that MTX has a negative effect on cognitive behavior related to hippocampal cell proliferation [22,23]. Although several mechanisms have been suggested to explain the cognitive impairment associated with MTX, the precise mechanisms remain poorly understood.

In this study, we examined the time- and dose-dependent changes in the number of Ki-67 (proliferating cell marker)- and doublecortin (DCX; immature progenitor neuron marker)-positive cells in the hippocampal dentate gyrus (DG) of adult C57BL/6 mice after MTX treatment to elucidate the detrimental effect of MTX on neurogenesis in the adult hippocampus. In addition, the effect of MTX on hippocampus-dependent behavioral dysfunction was estimated using a hippocampus-dependent learning paradigm (the object recognition memory test) and depression-like behavior test (the tail-suspension test). Furthermore, this study compared the detrimental effect of MTX treatment on 0-day *in vitro* (DIV) immature hippocampal cells with that of 14-DIV mature cells to elucidate the differential effect of MTX treatment on immature and mature hippocampal cells in an *in vitro* system.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice aged 8–9 weeks and pregnant Sprague–Dawley rats were obtained from a specific-pathogen-free colony at Oriental, Inc. (Seoul, Korea). The animals were housed in a room which was maintained at $23 \pm 2\,^{\circ}C$, with relative humidity of $50 \pm 5\%$, artificial lighting from 08:00 to 20:00 h, and 13–18 air changes every hour. The animals were given tap water and commercial rodent chow (Samyang Feed, Seoul, Korea) *ad libitum*. The Institutional Animal Care and Use Committee at Chonnam National University approved the protocols used in this study, and the animals were cared for in accordance with the Guidelines for Animal Experiments.

2.2. Drug treatment and tissue sampling

MTX (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in sterilized 0.9% saline. The time- and dose-dependent effects of MTX on neurogenesis in the adult mouse hippocampus were observed after administration of an intraperitoneal (i.p.) injection of MTX (0–200 mg/kg). The vehicle group was injected i.p. with 0.9% saline. The mice were sacrificed, and the hippocampi were dissected from each group at 6 h, 12 h, 24 h, 3 days, 7 days, and 14 days ($n = 3$ mice/group) after receiving an injection of MTX. The samples were processed for embedment in paraffin wax after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) using routine protocols.

2.3. Behavioral analysis

Behavioral dysfunction in mice who received MTX (i.p., 40 mg/kg) was measured by open-field analysis ($n = 6$ mice/group), an object recognition memory test ($n = 6$ mice/group), and a tail-suspension test ($n = 8$ mice/group) at 1 and 7 days after injection.

2.3.1. Open-field test

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

2.3.2. Object recognition memory test

The object-recognition memory test was used to examine hippocampus-dependent memory [24,25]. The test was similar to that described by Yang et al. [26]. Briefly, two randomly selected, differently shaped objects were presented to each mouse for 10 min during training. Twenty-four hours after training, another pair of objects (one old object and one novel object) was presented to the trained mice. If, for example, cube- and pyramid-shaped objects were presented during training, then a cylinder-shaped object was used as a novel object during testing. The interactions of the mouse with each object, including approaches and sniffing, were scored. If the mouse remembered an old object, it would show preference to the novel object during testing. The percentage of preference was defined as the number of interactions for a specific object divided by the total number of interactions for both objects.

2.3.3. Tail-suspension test

The tail-suspension test was similar to that described by Steru et al. [27]. 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 using the SMART video-tracking system (Panlab, Barcelona, Spain).

2.4. Primary hippocampal cell culture and treatment of cultures

The method used for primary hippocampal cell culture has been previously described in detail [28]. Briefly, hippocampi were dissected from embryonic Sprague–Dawley rats at 14–15 gestational days. After dissection, tissues were chopped and digested with 10 units/mL papain (Worthington, Freehold, NJ, USA) and 100 units/mL DNase I (Roche, Basel, Switzerland) in dissociation buffer at 37 °C for 30 min. The digestion was triturated with Neurobasal A medium (Invitrogen, Carlsbad, CA, USA). The cells were seeded at a density of 0.2×10^6 cells/well on poly-D-lysine hydrobromide (150 μg/mL; Sigma–Aldrich, St. Louis, MO, USA)-coated 24-well plates (NUNC™; Thermo Fisher Scientific, Pittsburgh, PA, USA). Two hours after plating, Neurobasal A was replaced with growth medium including Neurobasal A, 1× B27 supplement (Invitrogen), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.5 mM glutamine (Invitrogen). All cultures were kept at 37 °C and 5% CO_2. Cells were treated with 0, 40, 200, or 800 μM of MTX at 0 and 14 DIV. To evaluate the cytoprotective effects of the caspase-3-specific inhibitor Z-DEVD-FMK (5 μM; BioVision, Mountain View, CA, USA) on immature hippocampal neurons with MTX, the inhibitor was added 30 min before MTX treatment. The cells were assayed at 24 h after treatment.

2.5. Cell viability and cytotoxicity evaluation

Cell viability was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) assay. The assay was based on the reduction of MTT by living cells to yield a soluble formazan product that could be colorimetrically detected. MTT was added to cells in 4-well plates to 0.5 mg/mL

in culture medium. After a 2-h incubation at 37 °C with 5% CO_2, the resultant formazan crystals were dissolved in MTT solubilization solution (Sigma–Aldrich). The solubilized formazan products were quantified by an Emax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) for absorbance at a wavelength of 570 nm. The background absorbance was subtracted by spectrophotometric measurements at a wavelength of 690 nm.

Cytotoxicity was evaluated using a lactate dehydrogenase (LDH) release assay. A commercially available LDH-cytotoxicity assay kit from Biovision (Mountain View, CA, USA) was used as recommended by the manufacturer. The optical density values were quantified by a microplate reader (Emax, Molecular Devices) for absorbance at a wavelength of 490 nm.

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

The level of DNA fragmentation was detected using *in situ* nick end labeling (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, TUNEL), which was performed according to the manufacturer's instructions (ApopTag® *In Situ* Apoptosis Detection Kit; Invitrogen).

2.7. Immunohistochemistry

Five-micron-thick coronal sections were deparaffinized, hydrated, and allowed to react with immunohistochemical markers for proliferating cells and immature progenitor cells [*i.e.* 1:200 dilution of monoclonal rabbit anti-Ki-67 (DRM004; Acris Antibodies GmbH, Hiddenhausen, Germany) and 1:400 dilution of polyclonal rabbit anti-DCX (Cell Signaling Technology, Beverly, MA, USA) antibody], for 2 h. The sections were reacted with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit, Burlingame, CA, USA) for 45 min. Immunoreactivity was performed using the avidin–biotin peroxidase complex (Vector ABC Elite Kit) prepared according to the manufacturer's instructions for 45 min. The peroxidase reaction was developed using a diaminobenzidine substrate kit (SK-4100; Vector). As a control, the primary antibodies were omitted for a few test sections in each experiment. The sections were counterstained with Harris's hematoxylin before being mounted.

2.8. Cell counting

The number of cells showing specific characteristics of proliferating cells (immunopositive for Ki-67) and immature progenitor cells (immunopositive for DCX) in the hippocampus was scored by an observer blinded to the identity of the sample using a histomorphometric approach [29]. The brain from each mouse was sampled at approximately 2.12 mm behind the bregma. A standardized counting area that contained 5-μm-thick coronal sections in a 1-in-10 series of sections representing the rostral/mid-hippocampus was used. For each mouse, three nonoverlapping sections were analyzed, one from each of the three regions of the hippocampus (approximately 50 μm apart). All positively immunolabeled cells within the subgranular zone (SGZ) of the supra- and infrapyrimidal blades of the DG were quantified. The number of immunopositive cells was determined from the values obtained from each DG in the three brain sections. The mean number of immunopositive cells in the three sections of each mouse was taken as $n = 1$. The number of immunopositive cells was expressed as the mean ± SEM for each group ($n = 3$).

2.9. Double-immunofluorescence

Colocalization of the TUNEL reaction and active caspase-3 (Cell Signaling Technology) immunoreactivity was examined using double-immunofluorescent labeling in the same well at 14 DIV. In brief, after finishing the TUNEL reaction, which was allowed to react with fluorescein isothiocyanate-labeled anti-digoxigenin antibody, double immunofluorescence was applied using a 1:50 dilution of tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit IgG (Sigma–Aldrich) secondary antibodies to colocalize the TUNEL reaction and active caspase-3-immunoreactivity in the same well. The double-immunofluorescence-stained specimens were determined by immunofluorescence microscopy using a BX-40 apparatus (Olympus) with a ProgRes® CFscan digital camera (Jenoptik, Jena, Germany).

2.10. Western blotting

The medium was removed, and an SDS sample buffer (4×) was added to each culture. The cells were scraped and sonicated for 10 s, and the samples were heated to 100 °C for 10 min. The samples were then separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane, and the membranes were incubated with a 1:1000 dilution of either rabbit anti-cleaved (active) caspase-3 antibody (Cell Signaling Technology) or rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology). The immunoblot was performed as previously described [28]. The bands were quantified using Scion Image Beta 4.0.2 for Windows XP software (Scion, Frederick, ME, USA).

2.11. Statistical analysis

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

3. Results

3.1. Histological change in the mouse hippocampus following MTX injection

We first examined histological changes in hippocampal structure using hematoxylin and eosin (H&E) staining. No unusual hippocampal structures were observed in adult mice at 6 h to 14 days after acute MTX exposure (Fig. 1A and B). However, TUNEL staining revealed the presence of apoptotic nuclei in the hippocampal DG of the mice after exposure to MTX, whereas TUNEL-positive apoptotic cells were quite rare in the vehicle-treated controls (Fig. 1C and D). The number of TUNEL-positive apoptotic nuclei in the DG increased steeply from 0 to 12 h after acute exposure to MTX (Fig. 1D and E), reaching a maximum of 3.0 ± 0.58 nuclei/DG ($n = 3$, $p < 0.01$ *vs.* controls). After 12 h, the number of TUNEL-positive nuclei declined (Fig. 1E).

3.2. Change in Ki-67 and DCX expression in the hippocampal DG of adult mice following MTX injection

To elucidate the effect of MTX on adult hippocampal neurogenesis, two immunohistochemical markers of neurogenesis, the levels of Ki-67 (a proliferating cell marker) and DCX (an immature progenitor cell marker) in the DGs of hippocampi were semiquantitatively evaluated after MTX 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. 2A). The number of Ki-67-immunopositive cells sharply decreased between 0 [19.00 ± 1.53 nuclei/DG ($n = 3$)] and 12 h after MTX injection [11.33 ± 2.03 nuclei/DG ($n = 3$, $p < 0.05$)] and maintained

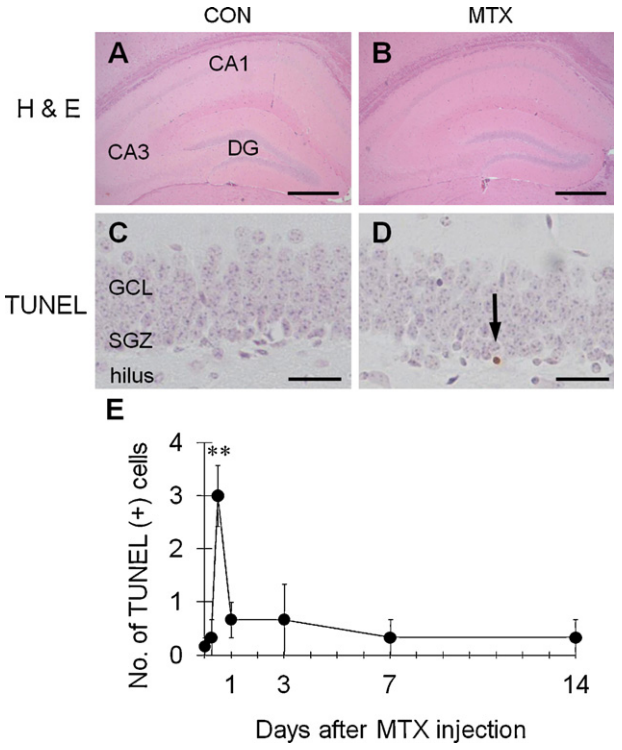

Fig. 1. Histological results for the hippocampi of vehicle-treated controls 24 h after injection with vehicle (A and C) and those of MTX-treated mice 24 h after administration of 40 mg/kg MTX (B and D). (A and B) No unusual hippocampal structure was observed in vehicle-treated controls or MTX-treated mice by hematoxylin and eosin (H&E) staining. (C and D) Apoptotic cells were detected by TUNEL staining. Although TUNEL-positive apoptotic nuclei were rarely detected in the DG regions of the hippocampus of vehicle-treated controls, apoptotic nuclei were detected by TUNEL staining in the SGZ of the DG of MTX-treated mice. The arrow in D indicates TUNEL-positive apoptotic nuclei. (E) Apoptotic cells in the SGZ of the DG adult mouse hippocampi changed in a time-dependent manner following injection of MTX. The number of apoptotic cells in the DG significantly increased 12 h after MTX injection. GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars in (A) and (B) represent 400 μm. Scale bars in (C) and (D) represent 30 μm. The data are reported as the mean ± SE (for E, $n = 3$ per group). $^{**}p < 0.01$ *vs.* vehicle-treated controls (24 h after treatment).

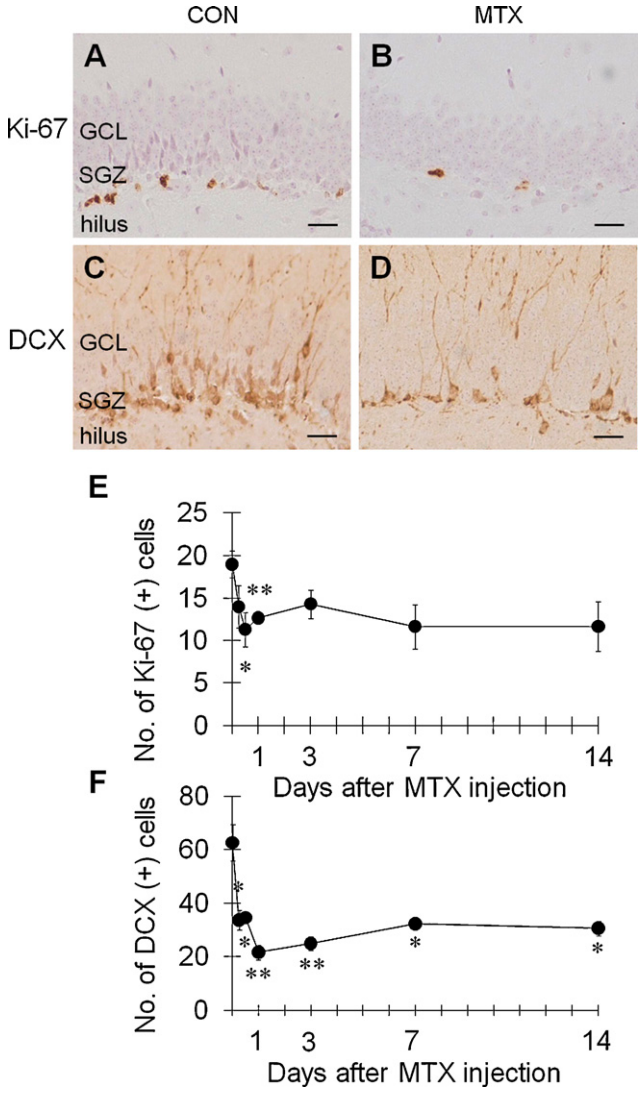

Fig. 2. Temporal profiles of Ki-67 and DCX expression in the DG of the adult hippocampus after MTX treatment. (A and B) Representative images showing Ki-67-positive cells in the DGs of adult hippocampi in vehicle-treated mice and MTX-treated mice (40 mg/kg) 24 h after injection. (C and D) Representative images showing DCX-positive cells in the DGs of adult hippocampi in vehicle-treated mice and MTX-treated mice (40 mg/kg) 24 h after injection. (E) The number of Ki-67-positive cells in the DG significantly decreased 12–24 h after MTX injection and maintained this low level at 14 days after MTX injection, although the decrease was not significant by 14 days. (F) The number of DCX-positive cells in the DG markedly decreased 6–24 h after MTX injection and significantly maintained this low level by 14 days after MTX injection. The cells were counterstained with hematoxylin (A–D). GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars in (A–D) represent 20 μm. The data are reported as the mean ± SE (for E and F, $n = 3$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* controls (0 h after treatment).

this low level at 14 days after injection, although the decrease was not significant at 14 days [11.67 ± 2.91 nuclei/DG ($n = 3$, $p = 0.089$)] (Fig. 2B and E). 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. 2C). The immunoreaction revealed a ramified body shape in the cells in the GCL. To quantify DCX-labeling, a cell was counted as DCX immunopositive if it had both immunopositively labeled cytoplasm and nuclei counterstained with hematoxylin (Fig. 2C). The number of DCX-immunopositive cells declined between 0 [62.67 ± 6.69 nuclei/DG ($n = 3$)] and 12 h after MTX injection [34.67 ± 2.03 nuclei/DG ($n = 3$, $p < 0.05$)], reaching the lowest number of DCX-positive cells at 24 h [21.67 ± 2.73 nuclei/DG ($n = 3$, $p < 0.01$)] (Fig. 2D and F). The number of labeled cells maintained this low level at 14 days after MTX injection [30.67 ± 2.73 nuclei/DG ($n = 3$, $p < 0.05$)] (Fig. 2F).

As shown in Fig. 3A, Ki-67 immunoreactivity in the SGZ of the DG progressively decreased with increasing dose of injected MTX (0–200 mg/kg). The number of Ki-67-positive proliferating cells sharply declined at 0–10 mg/kg MTX and continuously decreased as the dose increased. A decrease in the number of DCX-positive cells in the DG was also observed with increasing administration dose (Fig. 3B). A significant decrease was observed in the number of DCX-positive cells between 0 and 40 mg/kg, and the change leveled off at 40–100 mg/kg (Fig. 3B).

3.3. Impairment of hippocampus-related behaviors in adult mice following MTX injection

To evaluate hippocampal neurogenesis-related behavioral dysfunction in adult mice exposed to MTX, basal locomotor activity, depression-like behaviors, and learning and memory tasks were performed in adult mice at 1 and 7 days after MTX (40 mg/kg) injection.

We first examined basal locomotor activity of mice 1 and 7 days after MTX treatment (40 mg/kg) in a novel environment by performing open-field analysis ($n = 6$ per group). Open-field analysis quantified overall activity that could reflect the motivation and performance of the mice. The vehicle-treated controls and

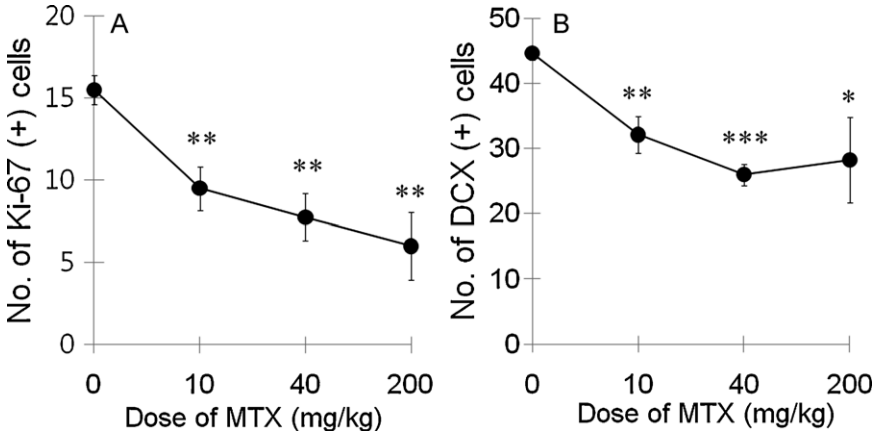

Fig. 3. Dose-dependent changes in Ki-67 and DCX immunoreactivity in the DGs of adult mouse hippocampi 24 h after MTX treatment. (A) The number of Ki-67-positive cells in the DGs. (B) The number of DCX-positive cells in the DGs. Ki-67 and DCX immunoreactivity in the SGZ of the DG decreased as the MTX dose increased (0–200 mg/kg). The data are reported as the mean ± SE ($n = 4$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* vehicle-treated controls.

the MTX-treated mice showed comparable ambulatory movement counts, moving distances, and ambulatory movement times and resting times at 1 and 7 days after injection (see Table 1). These data indicated that MTX administration did not alter locomotor activity in adult mice, suggesting normal movement of MTX-treated mice.

We examined MTX (40 mg/kg)-treated mice ($n = 6$ per group) by a sensitive hippocampus-dependent paradigm, object-recognition memory [24]. During the 10-min training session, vehicle- and MTX-treated mice showed no significant differences in interaction with the objects, indicating normal motivation and exploratory activity (data not shown). At 1 and 7 days after MTX injection, vehicle- and MTX-treated mice also displayed equal preference for the two objects during training (data not shown). During the test 24 h after training, the preferences (mean ± SEM) toward a novel object were 70.79 ± 2.31% and 70.34 ± 1.88% in the mice trained at 1 and 7 days after vehicle injection, respectively, and 65.19 ± 1.15% and 62.39 ± 1.02% in the mice trained at 1 and 7 days after MTX injection, respectively (Fig. 4A). Thus, MTX-treated mice showed memory deficits (1 day after injection, $p < 0.05$ *vs.* vehicle-treated controls; 7 days after injection, $p < 0.01$ *vs.* vehicle-treated controls) in object-recognition memory test.

The tail-suspension test has been recognized as a useful experimental paradigm for assessing depression-like behavior and the activity of antidepressants. This test was also performed at 1 and 7 days after MTX (40 mg/kg) injection. MTX-treated mice exhibited a longer duration of immobility during the test compared with vehicle-treated mice at 1 day (vehicle-treated control: 121 ± 8.95 s, MTX-treated mice: 165 ± 11.77 s; $n = 8$, $p < 0.01$) and 7 days (vehicle-treated control: 137.75 ± 8.70 s, MTX-treated mice: 165.25 ± 8.14 s; $n = 8$, $p < 0.05$) after injection (Fig. 4B). Thus, MTX-treated mice showed remarkable depression-like behaviors on the tail-suspension test.

3.4. Cytotoxicity and cell viability in hippocampal cells with MTX

We analyzed the cytotoxicity of cultured hippocampal cells induced 24 h after MTX treatment of 0–800 μM by an LDH release assay to assess whether there was a difference in cytotoxicity between 0-DIV immature and 14-DIV mature cells. At 0 DIV, cytotoxicity increased in a dose-dependent pattern within the range of treatment applied (Fig. 5A). At 14 DIV, however, MTX did not increase cytotoxicity (Fig. 5B). In addition, the cell viability estimated by the MTT assay decreased progressively with increasing doses of MTX in 0-DIV immature cells (Fig. 5C), whereas it was hardly changed in 14-DIV mature cells (Fig. 5D). This suggests that the MTX susceptibility of such hippocampal cells depends on their level of maturation.

3.5. Cell phenotypes of 0-DIV hippocampal culture

To confirm the cell phenotypes of 0-DIV hippocampal cultured cells, we performed double-immunofluorescent staining. We first evaluated nestin and DCX, which are immunohistochemical markers for neural stem cells and immature progenitor neurons, respectively, in 0-DIV hippocampal cells (Supplemental Fig. 1A). Nestin-positive and DCX-positive cells (mean ± SE of total cells; $n = 9$) constituted 70.05 ± 2.04% and 29.50 ± 1.27% of total cells in 0-DIV hippocampal cultured cells, respectively (Supplemental Fig. 1B). The double-immunofluorescence results showed that nestin-positive cells were slightly matched with DCX immunoreactivity (6.47 ± 3.08% of total cells; Supplemental Fig. 1B). Thus, 0-DIV hippocampal cultured cells have 93.09% ± 2.31% neural stem and/or progenitor cells in this experimental system.

Table 1
Open-field analysis of mice placed in a novel environment at 1 and 7 days after acute MTX (40 mg/kg) injection.

Parameters	1 day after injection		7 days after injection	
	Control	MTX	Control	MTX
Movement episodes	32.17 ± 2.63	28.33 ± 2.6	34.83 ± 2.46	35.67 ± 2.83
Distance (cm)	340.68 ± 28.05	403.78 ± 61.53	314.32 ± 31.76	379.08 ± 30.81
Movement time (s)	207.33 ± 7.08	223.67 ± 9.97	196.33 ± 15.39	204.5 ± 7.89
Rest time (s)	48.33 ± 5.7	40.33 ± 5.91	59 ± 13.03	55 ± 4.97

Open-field data for vehicle-treated controls (injected with 0.9% saline, i.p., $n = 6$) and MTX-treated mice (injected with MTX in 0.9% saline at 40 mg/kg, i.p., $n = 6$) at 1 and 7 days after injection. No significant differences were found in the number of ambulatory movement episodes ($p = 0.324$, 1 day after injection; $p = 0.829$, 7 days after injection), movement distance ($p = 0.373$, 1 day after injection; $p = 0.174$, 7 days after injection), ambulatory movement time ($p = 0.211$, 1 day after injection; $p = 0.647$, 7 days after injection), or resting time ($p = 0.353$, 1 day after injection; $p = 0.780$, 7 days after injection) between vehicle-treated controls and MTX-treated mice at 1 and 7 days after injection. The data are reported as the mean ± SEM.

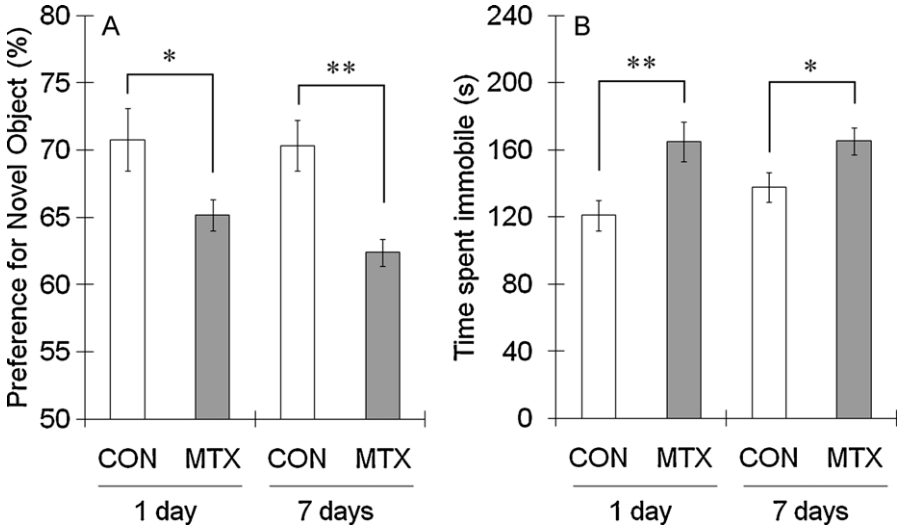

Fig. 4. Effect of MTX on learning and memory and depression-like behaviors. (A) Vehicle-treated controls (1 and 7 days after injection of only saline) and MTX-treated mice (1 and 7 days after 40 mg/kg MTX treatment) were examined ($n = 6$ per group) using an object-recognition memory test. A significant difference was observed in novel object preference between the vehicle-treated controls and MTX-treated mice during testing. (B) Vehicle-treated controls (1 and 7 days after injection of only saline) and MTX-treated mice (1 and 7 days after 40 mg/kg MTX treatment) were examined ($n = 8$ per group) using the tail-suspension test. In the tail-suspension test, the time spent immobile was significantly higher in MTX-treated mice than in the vehicle-treated control mice. The data are reported as the mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* vehicle-treated controls.

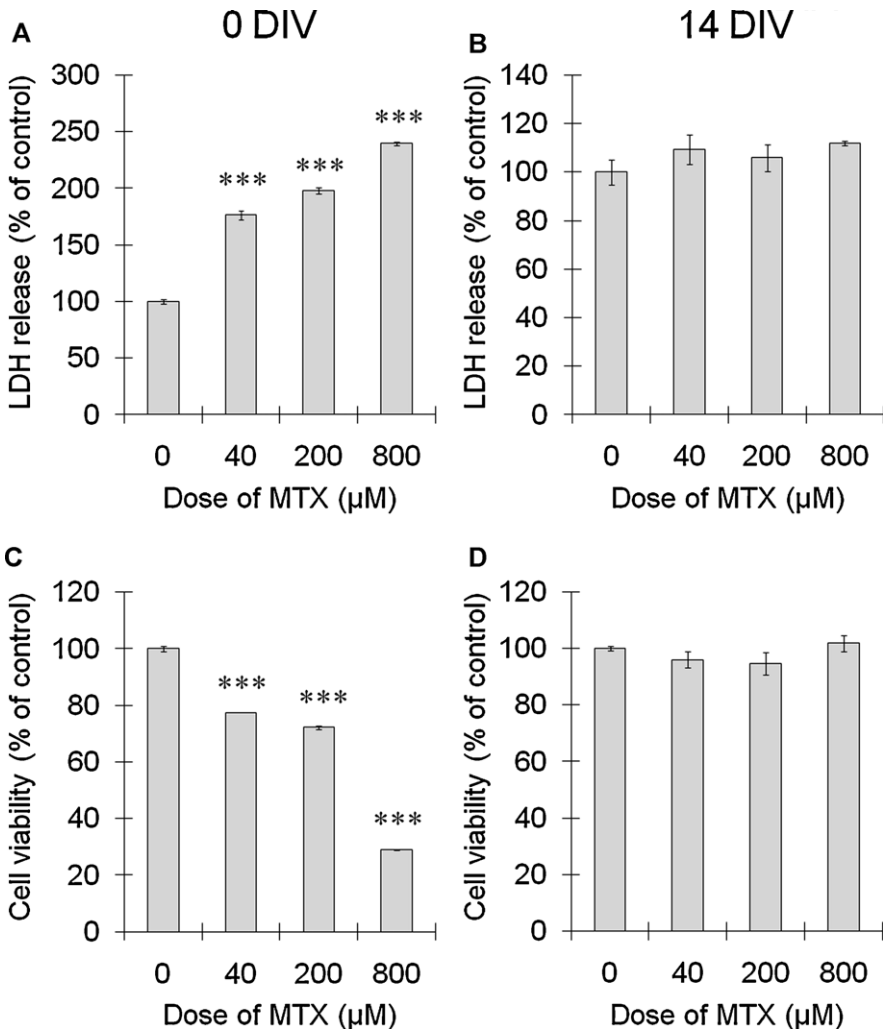

Fig. 5. The detrimental effect of MTX on cytotoxicity and cell viability in neural precursor cells. LDH release assay (A and B) and MTT assay (C and D) showing cytotoxicity and cell viability of hippocampal cells, respectively, evaluated 1 day after treatment at 0 (A and C) and 14 DIV (B and D). At 0 DIV, the amount of LDH released in the medium significantly increased (A), and the cell viability measured by MTT assay significantly declined (C) in a dose-dependent manner within the range of treatment applied (0–800 μM). In contrast, at 14 DIV, cell viability was not changed in either the LDH release assay or the MTT assay. The data are reported as the mean ± SE. $n = 3$ cultures per condition. $^{***}p < 0.001$ *vs.* vehicle-treated controls.

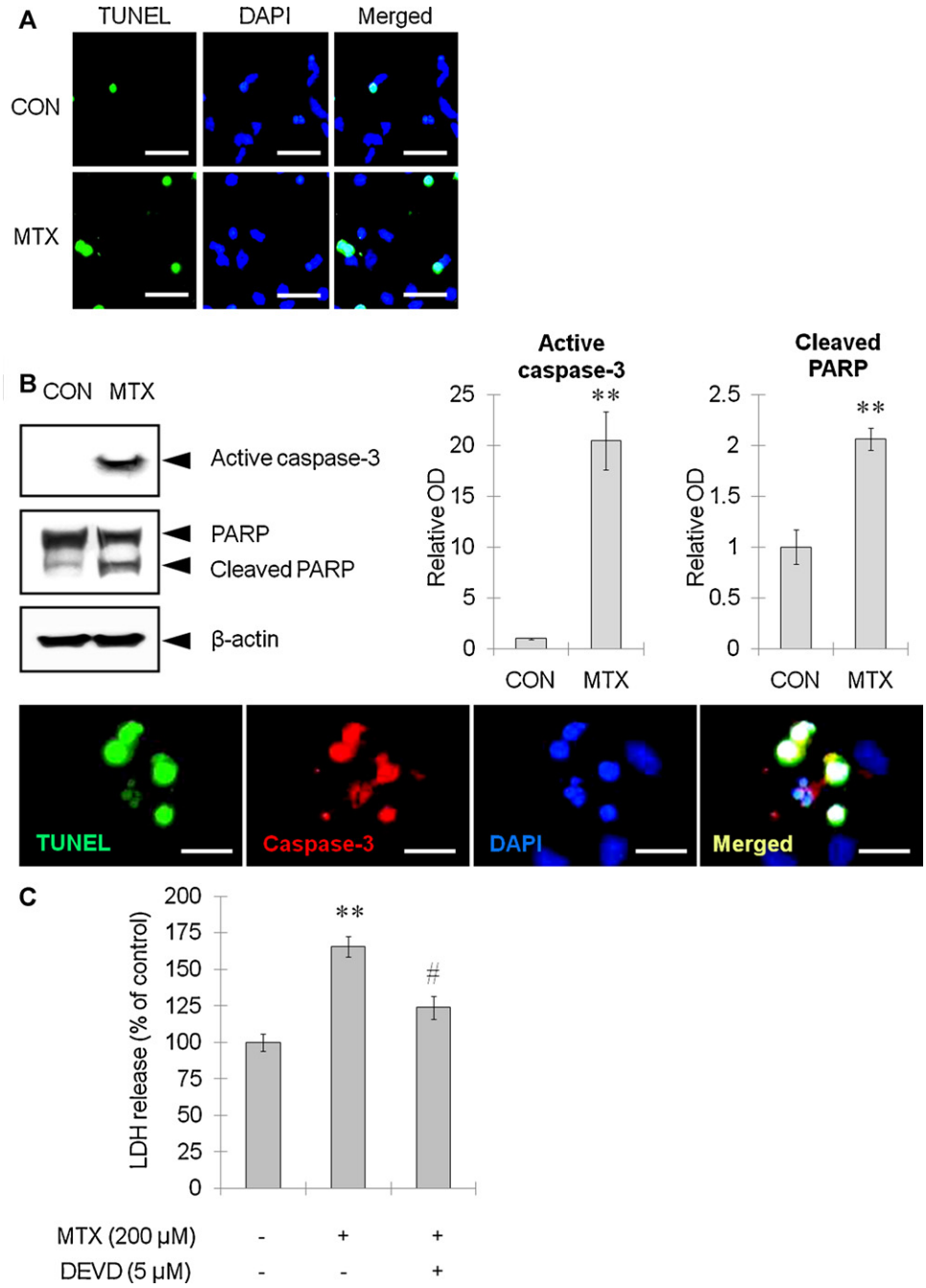

Fig. 6. Caspase-dependent apoptotic pathway after MTX treatment. (A) TUNEL-positive (green) apoptotic cell death was observed more in neural precursor cells after MTX (200 μM) treatment. (B) The upper panel shows Western blot analysis indicating active caspase-3 and cleaved PARP expression in neural precursor cells (upper panel, left). The relative expression levels of active caspase-3 and cleaved PARP were significantly increased in MTX-treated cells (200 μM) compared with vehicle-treated cells (upper panel, right). The lower panel shows TUNEL (green) and caspase-3 (red) detection in neural precursor cells after MTX treatment. The double-immunofluorescent results showed that TUNEL-positive cells were mostly overlapped with caspase-3-positive cells. (C) MTX-treated cells showed significantly increased LDH release in the medium. However, pretreatment with Z-DEVD-FMK (caspase-3-inhibitor) significantly inhibited LDH release in the medium in MTX-treated cells (200 μM). Scale bars in A = 40 μm. Scale bars in B = 15 μm. The data are reported as the mean ± SE. $n = 3$ cultures per condition. $^{**}p < 0.01$ *vs.* vehicle-treated controls. $\#p < 0.05$ *vs.* MTX-treated controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.6. MTX induces caspase-3-dependent cell death in immature hippocampal cells

TUNEL-positive apoptosis was markedly enhanced in MTX-treated immature cells (Fig. 6A). To determine whether MTX-treated cell death in immature hippocampal neurons was associated with the caspase-dependent pathway, caspase-3 activation and caspase-specific PARP cleavage were assessed by Western blot and double-immunofluorescence (Fig. 6B). At 0 DIV, active caspase-3 and cleaved PARP expression were significantly increased 24 h after MTX treatment (200 μM) (Fig. 6B; upper panels). Additionally, TUNEL-positive cells were colocalized with active caspase-3 in MTX-treated immature hippocampal cells (Fig. 6B; lower panel). Furthermore, caspase-3-specific inhibitor Z-DEVD-FMK significantly blocked LDH release from immature hippocampal cells with MTX treatment (Fig. 6C). This suggests that MTX-induced cell death in immature hippocampal cells depends on a proapoptotic caspase pathway.

4. Discussion

This study demonstrated the acute administration of MTX induced apoptosis and reduced the number of Ki-67 (proliferating cells)- and DCX (immature progenitor neurons)-positive cells in the adult mouse hippocampus and also induced hippocampal dysfunction, as reflected in cognitive impairment and depression-like behavior. Moreover, our *in vitro* study revealed that MTX exposure induced apoptotic cell death in immature hippocampal cultured cells through a caspase-dependent pathway, but did not in mature hippocampal cells.

In the adult brain, progenitor/stem cells generate new neural cells in the DG of the hippocampus [30,31]. The hippocampus is one of several limbic structures that have been extensively studied in individuals with learning and memory difficulties and depression. Various drugs, as well as hormones and growth factors, can regulate the rate of cell proliferation in the hippocampus and influence the behaviors controlled by this brain region [32]. Inhibition of hippocampal neurogenesis with toluene exposure, ionizing radiation, and cyclophosphamide exposure has been shown to cause cognitive impairment and/or depression in adult mice [24,26,33]. In this study, MTX induced an increase in apoptosis and a decrease in neurogenesis in the DG of the adult hippocampus. These results are consistent with those of previous studies in which animals treated with MTX showed a decrease in hippocampal cell proliferation [23]. Therefore, patterns of memory ability and mental activity may be associated with alterations in hippocampal neurogenesis. However, further studies are needed to establish an unequivocal link between the change in hippocampal neurogenesis and hippocampal neurogenesis-dependent memory impairment and depression caused by chemotherapy.

Cognitive impairment and mental depression occur in a subset of cancer survivors, and the symptoms are generally subtle. Most evidence suggests an association with chemotherapy, although other factors related to the diagnosis and treatment of cancer may contribute [34]. Several clinical and experimental studies have reported cognitive impairment to be a side effect of cancer chemotherapy in cancer patients and experimental animals. Previous studies using mouse models reported that a combination of MTX (37.5 mg/kg) and 5-fluorouracil (75 mg/kg) induced cognitive deficits in spatial memory, non-matching-to-sample (NMTS) learning, and a delayed-NMTS test of nonspatial memory [22]. Additionally, using novel-location recognition testing, Lyons et al. [35] found that MTX chemotherapy causes cognitive impairments and a reduction in both proliferation and survival of neural precursors in the hippocampus. These results are similar to those of this study, which found MTX-induced cognitive deficits at 1 and 7 days after treatment in object-recognition memory tests associated with the reduction in hippocampal neurogenesis. In addition, in clinical cases, the prevalence of anxiety and/or depression was higher in cancer patients undergoing chemotherapy than in those receiving other treatments [36]. Similar to clinical cases, the present study found remarkable depression-like behaviors on the tail-suspension test after acute MTX administration in adult mice. Mental depression and cognitive impairment may be due not only to changes in neurotransmitter concentrations and receptor activity levels but also to impairment of brain plasticity and tissue remodeling and alterations in adult hippocampal neurogenesis [37–40]. Therefore, as shown in this study, the detrimental effects of MTX exposure, such as the reduction in hippocampal neurogenesis and the induction of apoptosis, may induce hippocampus-dependent behavioral dysfunction, including memory deficits and depression.

Folate is critical to normal brain development and to optimal cognitive function in the mature brain. It has been shown to play critical roles in the metabolism of the neurotoxic amino acid homocysteine, neurotransmitter synthesis, and methylation reactions necessary for the maintenance of the myelin sheath [41]. Poor folate status is increasingly recognized as an important and potentially modifiable risk factor for age-related cognitive decline and impairment in elderly people [42]. Low circulating folate has been shown to be significantly associated with mild cognitive impairment and depression in nondemented populations [43,44], suggesting that it increases the risk of cognitive decline and depression. Folate-deficient cognitive impairment corresponded to altered phosphatidylcholine and choline metabolism [45]. Dietary folic acid deficiency dramatically increased blood homocysteine levels and significantly reduced the number of proliferating cells in the DG of the hippocampus in adult mice [46]. MTX inhibited folate-dependent biochemical processes, thus inhibiting DNA synthesis [9]. Li et al. [41] reported that both acute and chronic MTX administration was associated with a marked reduction in serum and cerebrospinal fluid folate. Thus, we suggest that MTX-induced hippocampus-dependent behavioral dysfunction, such as cognitive impairment and depression, as well as a reduction in hippocampal neurogenesis, may be related to the reduction of folate by MTX treatment.

In addition, we examined the *in vitro* neurotoxicity of MTX in immature and mature hippocampal cells. There is a consensus that normal neural progenitor cells are exceptionally vulnerable to the toxic effects of various chemotherapeutic agents [47]. Cultured hippocampal cells were shown to form functional synapses and a network after 3–5 DIV [48]. In the present study, 0-DIV cultured hippocampal cells had approximately 93% neural stem and/or progenitor cells in this experimental system. Therefore, we chose to apply MTX to 0-DIV cultured hippocampal cells, which are comparable to neural stem/progenitor cells before cell connection and synapse formation. Many neuropathological conditions in the CNS are characterized by neuronal apoptosis [49]. Previous studies reported that many chemotherapeutic drugs, such as cisplatin, lomustine, topotecan, cyclophosphamide, and vincristine, induced cell death in cerebellar granule neurons or cortical neurons in 7–8-day-old Sprague–Dawley rat pups [50]. The reduction in survival is at least partially due to apoptosis [14,50]. The radiosensitivity of hippocampal cells depends on their differentiation [28]. These sensitivities of immature hippocampal cells are similar to those found in our study. In this study, the cell viability, using MTT assay, and cytotoxicity, using LDH assay, were shown to be reduced and increased, respectively, in a dose-dependent manner within the range of treatment applied at 0 DIV, but not at 14 DIV. Therefore, these results suggest that 0-DIV hippocampal immature cells were susceptible to MTX, but 14-DIV hippocampal mature cells were resistant to MTX. Furthermore, cultures of 0-DIV hippocampal cells provide a useful tool for the analysis of critical mechanistic parameters associated with the MTX treatment response of the brain and may provide a better understanding of the molecular changes in MTX-induced apoptosis of neural cells. Additionally, during the caspase-dependent apoptotic process, PARP is proteolytically cleaved and inactivated by caspase-3, a process that is widely used as an apoptotic marker [51]. In the present study, active caspase-3 and cleaved PARP expressions were significantly increased after treatment with MTX, and pretreatment with a caspase-3-inhibitor blocked hippocampal cell death, suggesting that MTX-induced apoptosis in immature hippocampal cells depends on a proapoptotic caspase pathway.

In conclusion, this *in vivo* study suggests that the number of proliferating and migrating cells in the DGs of hippocampi in adult C57BL/6 mice was significantly reduced after MTX treatment. The lower rate of hippocampal neurogenesis may be causally related to hippocampal dysfunction, such as depression and cognitive impairment, in adult mice. Moreover, this *in vitro* study has confirmed that the susceptibility of immature hippocampal cells

to MTX treatment is significantly higher than that of mature cells, indicating that the susceptibility of such hippocampal cells depends on their level of maturation. In addition, MTX treatment may induce caspase-dependent apoptosis in immature hippocampal cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.03.020.

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