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Temporal profiles of synaptic plasticity-related signals in adult mouse hippocampus with methotrexate treatment[☆]

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Abstract

Methotrexate, which is used to treat many malignancies and autoimmune diseases, affects brain functions including hippocampal-dependent memory function. However, the precise mechanisms underlying methotrexate-induced hippocampal dysfunction are poorly understood. To evaluate temporal changes in synaptic plasticity-related signals, the expression and activity of N-methyl-D-aspartic acid receptor 1, calcium/calmodulin-dependent protein kinase II, extracellular signal-regulated kinase 1/2, cAMP responsive element-binding protein, glutamate receptor 1, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor were examined in the hippocampi of adult C57BL/6 mice after methotrexate (40 mg/kg) intraperitoneal injection. Western blot analysis showed biphasic changes in synaptic plasticity-related signals in adult hippocampi following methotrexate treatment. N-methyl-D-aspartic acid receptor 1, calcium/calmodulin-dependent protein kinase II, and glutamate receptor 1 were acutely activated during the early phase (1 day post-injection), while extracellular signal-regulated kinase 1/2 and cAMP responsive element-binding protein activation showed biphasic increases during the early (1 day post-injection) and late phases (7–14 days post-injection). Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor expression increased significantly during the late phase (7–14 days post-injection). Therefore, methotrexate treatment affects synaptic plasticity-related signals in the adult mouse hippocampus, suggesting that changes in synaptic plasticity-related signals may be associated with neuronal survival and plasticity-related cellular remodeling.

Key Words

hippocampus; methotrexate; neurotrophic factor; synaptic plasticity-related signal; neural regeneration

Research Highlights

(1) Methotrexate altered the activities of synaptic plasticity-related signals and induced a significant increase in the expression of neurotrophic factors in adult mouse hippocampus. (2) Changes in synaptic plasticity-related signals and neurotrophic factors are associated with neuronal survival and plasticity-related cellular remodeling.

Abbreviations

NMDAR1, N-methyl-D-aspartic acid receptor 1; CaMKII, calcium/calmodulin-dependent protein kinase II; ERK1/2, extracellular signal-regulated kinase 1/2; CREB, responsive element-binding protein; GluR1, glutamate receptor 1; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor

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INTRODUCTION

Adjuvant chemotherapy is frequently used to treat cancer. The survival rate of cancer patients treated with cytostatic chemotherapy is high; however, treatment is associated with side effects including cognitive impairments in attention/concentration, speed of information processing, and memory^[1-4]. Furthermore, more patients treated with high-dose chemotherapy than patients treated with standard-dose chemotherapy exhibit deficits in cognitive performance as compared with healthy control subjects^[5-6].

Methotrexate is a cytostatic drug that is frequently used in adjuvant chemotherapy for breast cancer^[7]. It exerts anti-neoplastic effects by competitively inhibiting folate-dependent biochemical processes, thus resulting in the inhibition of DNA synthesis^[8]. However, methotrexate appears to be potentially more noxious than other agents and is associated with acute and chronic neurotoxicity because it easily passes through the blood-brain barrier^[9]. Several studies have reported that methotrexate negatively affects cognitive behavior, depression, and hippocampal neurogenesis^[10-12].

Neuronal synaptic plasticity in the adult brain is manifested at the cellular level by changes in dendritic growth, axonal sprouting, synaptic remodeling, and the creation of new synapses, and is closely linked with neurogenesis^[13]. Many studies have demonstrated that neurotoxic agents

and neurodegenerative diseases induce changes in neurogenesis and synaptic plasticity in the adult hippocampus^[14-15]. Although several mechanisms have been suggested to explain the cognitive impairments associated with methotrexate, little is known regarding the precise mechanisms underlying the deteriorative effects of such chemotherapeutic agents on brain function, particularly effects on synaptic plasticity. In this study, the temporal expression and activity of N-methyl-D-aspartic acid receptor 1 (NMDAR1), calcium/calmodulin-dependent protein kinase (CaMKII), extracellular signal-regulated kinase 1/2 (ERK1/2), cAMP responsive element-binding protein (CREB), glutamate receptor 1 (GluR1), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) were examined after methotrexate intraperitoneal injection (40 mg/kg) in the hippocampi of adult C57BL/6 mice to evaluate temporal changes in synaptic plasticity-related signals.

RESULTS

Changes in synaptic plasticity-related signals in the hippocampus of adult mice following methotrexate injection

Methotrexate administration led to significant perturbations in synaptic plasticity-related signals in the hippocampus (Figure 1, Table 1).

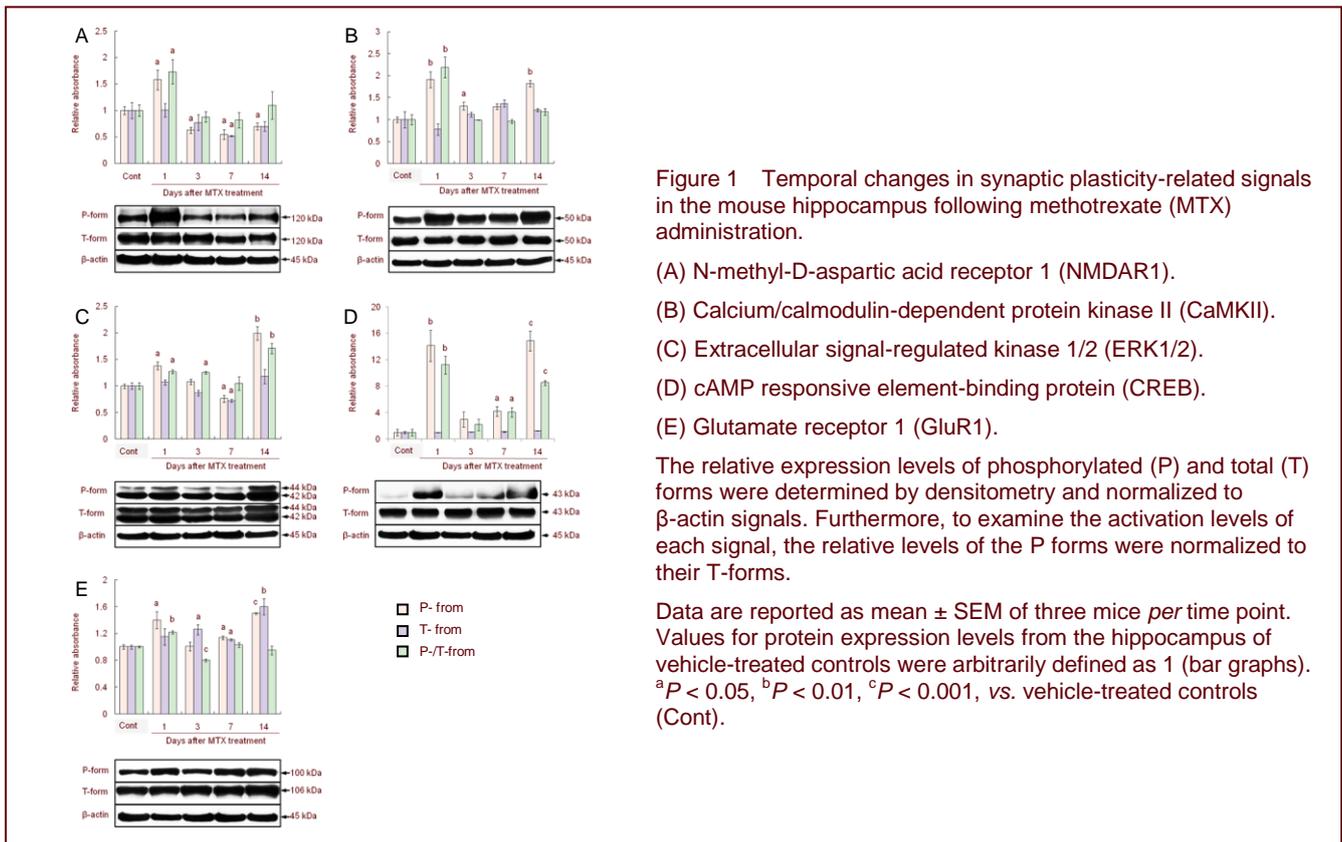


Table 1 Temporal changes in synaptic plasticity-related signals in the adult hippocampus after MTX treatment

Signals	Days after MTX (40 mg/kg) treatment			
	1	3	7	14
NMDAR1 Phosphorylated form	↑ ^a	↓ ^a	↓ ^a	↓ ^a
Total form	—	—	↓ ^a	—
Phosphorylated/total form	↑ ^a	—	—	—
CaMKII Phosphorylated form	↑ ^b	↑ ^a	—	↑ ^b
Total form	—	—	—	—
Phosphorylated/total form	↑ ^b	—	—	—
ERK1/2 Phosphorylated form	↑ ^a	—	↓ ^a	↑ ^b
Total form	—	—	↓ ^a	—
Phosphorylated/total form	↑ ^a	↑ ^a	—	↑ ^b
CREB Phosphorylated form	↑ ^b	—	↑ ^a	↑ ^c
Total form	—	—	—	—
Phosphorylated/total form	↑ ^b	—	↑ ^a	↑ ^c
GluR1 Phosphorylated form	↑ ^a	—	↑ ^a	↑ ^c
Total form	—	↑ ^a	↑ ^a	↑ ^b
Phosphorylated/total form	↑ ^b	↓ ^c	—	—

“—” means no significant increase or decrease compared with vehicle-treated controls; ↑ increase; ↓ decrease, ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001. vs. vehicle-treated controls.

MTX: Methotrexate; NMDAR1: N-methyl-D-aspartic acid receptor 1; CaMKII: calcium/calmodulin-dependent protein kinase II; ERK1/2: extracellular signal-regulated kinase 1/2; CREB: cAMP responsive element-binding protein; GluR1: glutamate receptor 1.

The level of phosphorylated NMDAR1 increased markedly at 1 day post-injection, but decreased significantly during 3–14 days post-injection as compared with vehicle-treated controls. Total NMDAR1 levels decreased sig-

nificantly at 7 days post-injection and the level of the phosphorylated/total form of NMDAR1 increased significantly at 1 day post-injection and then decreased 3–14 days post-injection (Figure 1A). The levels of phosphorylated CaMKII increased significantly 1–3 days post-injection and markedly increased again at 14 days post-injection. The levels of the phosphorylated/total form of CaMKII increased at 1 day post-injection (Figure 1B). The levels of phosphorylated ERK1/2 increased significantly at 1 day post-injection, decreased significantly at 7 days post-injection, but then markedly increased again at 14 days post-injection. Total ERK1/2 levels decreased significantly at 7 days post-injection. The level of the phosphorylated/total form of ERK1/2 increased significantly 1–3 days post-injection and markedly increased again at 14 days post-injection (Figure 1C). The levels of phosphorylated CREB and the phosphorylated/total form of CREB increased sharply at 1 day post-injection, decreased at 3 days post-injection, and then significantly increased again 7–14 days post-injection (Figure 1D). The levels of phosphorylated GluR1 increased significantly 1 and 7–14 days post-injection. The levels of total GluR1 increased significantly 3–14 days post-injection; however, the levels of the phosphorylated/total form of GluR1 increased significantly only at 1 day post-injection (Figure 1E).

Immunohistochemical analysis of plasticity-related signals in the mouse hippocampus following methotrexate injection (Figure 2)

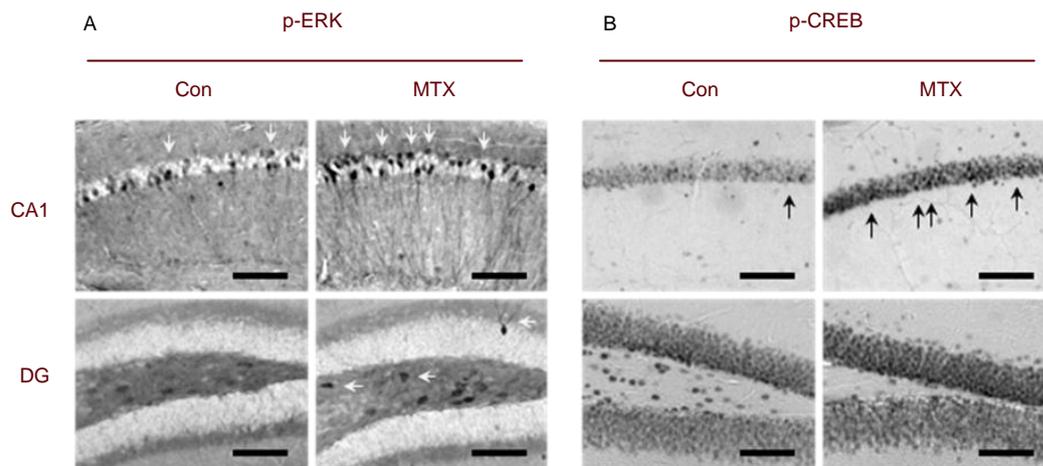


Figure 2 Representative images showing the immunoreactivities of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated responsive element-binding protein (p-CREB) in vehicle-treated and methotrexate (MTX)-treated mouse hippocampi at 14 days after injection.

(A) Phosphorylated ERK1/2. p-ERK1/2-positive cells (white arrows) were found predominantly in CA1 pyramidal neurons and the granular cell layer of the dentate gyrus (DG). This immunoreactivity increased after MTX injection in the cell bodies and dendrites of CA1 pyramidal cells and in DG granule cells.

(B) CREB. Low intensity CREB phosphorylation (black arrows), which was constitutively observed in nuclei of CA1 pyramidal neurons and DG granule cells, was increased after MTX injection. Scale bars represent 100 μm. CON: Control.

Changes in the phosphorylated form of plasticity-related signals, including ERK1/2 and CREB, after methotrexate injection were confirmed using immunohistochemistry. In the control hippocampus, constitutive low-intensity ERK phosphorylation was observed in both dendrites and cell bodies along the pyramidal layer of the *cornu ammonis* (CA) regions and the dentate gyrus (DG) granular cell layer (Figure 2A; left panel). Consistent with western blot results, phosphorylated ERK1/2, which was strongly expressed in mossy fibers, was increased in cells in the dendritic field of CA1 as well as the dentate gyrus (especially the hilus) at 1 and 14 days after methotrexate injection (Figure 2A; right panel). Constitutive low-intensity CREB phosphorylation was localized in nuclei of pyramidal cells in the CA regions and granular cells in the DG of controls (Figure 2B; left panel). Phosphorylated CREB immunoreactivity was also significantly increased at 1 and 14 days after methotrexate injection compared with controls (Figure 2B; right panel). The immunoreactivities of phosphorylated NMDAR1, CaMKII and GluR1 were also increased at 1 day after methotrexate injection compared with controls (data not shown).

Changes in neurotrophic factors in the mouse hippocampus following methotrexate injection

Temporal BDNF and GDNF expression were evaluated after methotrexate treatment to elucidate changes in neurotrophic factors in the adult hippocampus following methotrexate administration (Table 2, Figure 3). BDNF expression levels increased significantly 7–14 days

post-injection (Figure 3A). GDNF expression levels increased gradually 7–14 days after methotrexate treatment (Figure 3B).

Table 2 Temporal changes in neurotrophic factors in the adult hippocampus after methotrexate treatment

Signal	Days after methotrexate (40 mg/kg) treatment			
	1	3	7	14
BDNF	–	–	↑ ^b	↑ ^a
GDNF	–	–	↑ ^a	↑ ^a

“–” means no significant increase or decrease compared with vehicle-treated controls; ↑ increase; ^a*P* < 0.05 and ^b*P* < 0.01 vs. vehicle-treated controls. BDNF: Brain-derived neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor.

DISCUSSION

This is the first demonstration that methotrexate administration induces temporal changes in various synaptic plasticity-related signals in the adult mouse hippocampus. The activities of NMDAR1, CaMKII, and GluR1 increased acutely during the early phase, but ERK1/2 and CREB showed biphasic (both early and late phase) increases in the hippocampus following methotrexate administration. Additionally, the levels of neurotrophic factors including BDNF and GDNF increased significantly during the late phase of methotrexate administration (7–14 days post-injection).

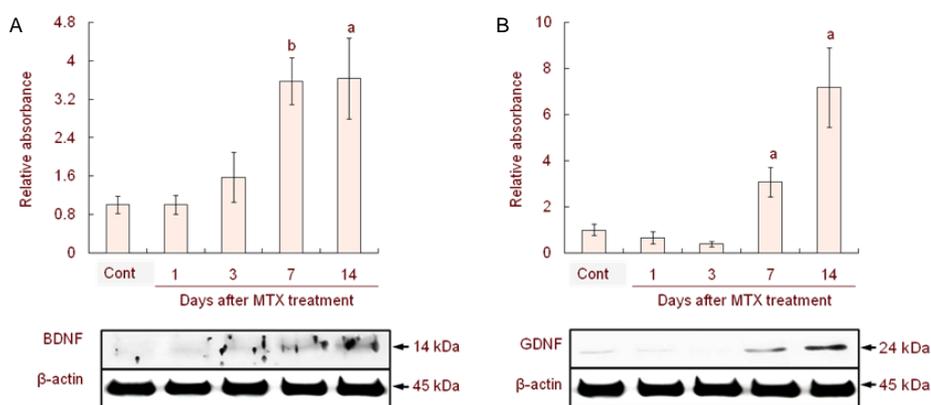


Figure 3 Time-response changes of neurotrophic factors in the mouse hippocampus following administration of methotrexate (MTX).

(A) Brain-derived neurotrophic factor (BDNF).

(B) Glial cell line-derived neurotrophic factor (GDNF).

The relative expression levels of proteins were determined by densitometry and normalized to β-actin signals. Data are reported as mean ± SEM. *n* = 3 mice *per* time point. Values for protein expression levels from the hippocampus of vehicle-treated controls (Cont) were arbitrarily defined as 1 (bar graphs). ^a*P* < 0.05, ^b*P* < 0.01 vs. vehicle-treated controls.

Methotrexate, a folate antagonist, is used to treat many malignancies and autoimmune diseases^[8]. However, several clinical and experimental studies have reported cognitive impairment as a side effect of methotrexate in cancer patients and in experimental animals^[10-12]. A previous study of a mouse model showed that methotrexate induces hippocampal dysfunction, including cognitive impairment, depression-like behavior, and a related decrease in neurogenesis^[12]. In addition, methotrexate acutely induces cell death in a small number of progenitor neural cells within the subgranular zone of the DG 12 hours after injection^[12]. In the adult and aging hippocampus, cognition is strongly associated with synaptic plasticity and hippocampal neurogenesis^[16]. Synaptic plasticity is thought to be a cellular learning and memory mechanism by which the brain is able to react to environmental stimuli. Calcium (Ca^{2+})-stimulated protein kinases may play a major role in regulating plasticity in stimulated neurons. Activation and plasticity-related gene expression of the NMDA receptor are related to many forms of learning-related plasticity in various brain areas^[17-18], and Ca^{2+} influx through NMDA receptors activates MEK-ERK1/2 signaling. Activation of ERK1/2 may further up-regulate CREB and CREB-mediated gene transcription. Additionally, CaMKII phosphorylated by Ca^{2+} influx acts as an important regulator by serving as an upstream molecule of ERK1/2 in the CREB signaling cascade^[19]. CREB functions as an important regulator of cellular proliferation, differentiation, and apoptosis and has been implicated as a regulatory factor in the survival and maturation of newly generated cells in the hippocampus^[20-21]. Although CREB activity mainly contributes to survival, aberrant CREB expression results in apoptosis in various cells^[22]. Low-level exposure to ionizing radiation, a cancer therapy, activates diverse plasticity-related proteins, which are critical for neuronal survival, synaptic plasticity, neurogenesis, and regulation of apoptosis, although it decreases the number of doublecortin-positive immature progenitor neurons^[23]. In this study, methotrexate induced early and/or late increases in various plasticity-related proteins in the adult mouse hippocampus. During the biphasic increases, methotrexate itself may lead to the increase in various plasticity-related signals in the first phase and consequently induce activation of CREB. Furthermore, most of the increase in phosphorylated ERK and CREB after methotrexate injection occurred in cells in CA1 and/or DG, where cell death was not observed. These results support the possibility that surviving cells activate plasticity-related signals that lead to an increase in neuronal survival. Here, we suggest two possibilities for the increase in various plasticity-related signals during the early phase that are associated with CREB activation. First, changes in plasticity-related signals

during the early phase may be related to the cytostatic effects of methotrexate on hippocampal progenitor neurons. Previous studies have reported that overexpression of NMDAR1 and other signals, and aberrant upregulation of CREB result in cell death^[22, 24]. In addition, whole-brain irradiation leads to relatively higher levels of the NMDAR1 and NMDAR2A subunits compared with the NMDAR2B subunits and thus results in altered synaptic transmission, reduced plasticity, and ultimately spatial learning and memory impairment^[25]. Thus, these phenomena may occur acutely after methotrexate injection and induce a reduction in hippocampal neurogenesis and hippocampal dysfunction such as memory impairment^[12, 26]. The second possibility is that the surviving hippocampal neurons activate plasticity-related signals to increase neuronal survival, as most hippocampal neurons in the CA regions, which comprise most of the hippocampus, survived methotrexate insult, as shown previously^[12]. However, further studies are needed to clarify the precise relationship between hippocampal dysfunction and changes in synaptic plasticity-related signals after methotrexate administration.

The ERK-CREB pathway, which is related to neuronal survival and memory function, may also underlie the production of neurotrophic factors such as BDNF^[27-28]. Neurotrophic factors play important roles in brain development, adult neurogenesis, and learning and memory^[29-31]. BDNF plays an important role in mediating neurogenesis, synaptic plasticity, and cell survival, and is crucial to learning and memory processes^[32]. BDNF activates CREB during remodeling to promote the expression of genes that prompt the formation, elongation, and stretching of dendritic spines^[33]. Another important neurotrophic factor, GDNF, promotes the proliferation of glial precursors and enteric neurons from the peripheral nervous system in culture, and increases cell genesis in the granule cell layer of the hippocampus^[34-35]. In this study, BDNF and GDNF increased gradually on days 7–14 after methotrexate administration. This increase in neurotrophic factors may be attributed to remodeling of hippocampal dendritic spines and activity of plasticity-related proteins during the late phase of methotrexate treatment.

Methotrexate competitively inhibits folate-dependent biochemical processes and DNA synthesis^[8]. However, in the present study, methotrexate stimulated the phosphorylation of synaptic plasticity-related signals. Previous studies have shown that ionizing radiation induces not only a reduction in hippocampal neurogenesis and cognitive impairment, but also changes in plasticity-related signals important for neuronal survival and the regulation of apoptosis^[23, 36]. Cyclophospha-

midate, a chemotherapeutic drug, also induces cognitive impairment after injection, and long-term potentiation is improved during recovery^[37-38]. Even though our previous study revealed that methotrexate decreases the rate of hippocampal neurogenesis and hippocampal function^[12], the present study suggests that synaptic plasticity-related signals may be increased as a compensatory mechanism against acute cell death or the inhibition of protein synthesis by methotrexate injection. In conclusion, the present results suggest that methotrexate-induced changes in synaptic plasticity-related signals show a biphasic pattern, which may initially be related to the effect of methotrexate itself and the remodeling processes due to the activity of neurotrophic factors. Based on these results, we suggest that methotrexate-induced changes in synaptic plasticity-related signals may be associated with neuronal survival and cellular remodeling related to synaptic plasticity in the adult mouse hippocampus.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the Laboratory of Veterinary Anatomy, Chonnam National University, Gwangju, South Korea, from 2009 to 2011.

Materials

Thirty male C57BL/6 mice, aged 8–9 weeks, were obtained from a specific-pathogen-free colony at Orient, Inc. (Seoul, South Korea). The animals were cared for in accordance with the internationally accepted principles for laboratory animal use and care as found in the NIH guidelines (USA).

Methods

Drug treatment and tissue sampling

Methotrexate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterilized 0.9% saline. Temporal changes in synaptic plasticity-related signals and neurotrophic factors in the hippocampus were observed after a single intraperitoneal injection of methotrexate (40 mg/kg). The vehicle group was injected intraperitoneally with 0.9% saline. The mice were sacrificed, and hippocampi were dissected from each group at 1, 3, 7, and 14 days ($n = 6$ mice/group) after injection. The hippocampal samples were stored at -80°C until western blot analysis and stored in 30% sucrose after fixation in 4% paraformaldehyde in PBS (pH 7.4) for immunohistochemistry.

Western blotting

The hippocampus from each mouse was individually immersed quickly in buffer H (50 mM β -glycerophosphate, 1.5 mM ethylene glycol tetraacetic acid, 0.1 mM Na_3VO_4 , 1 mM dithiothreitol, 10 $\mu\text{g}/\text{mL}$ aprotinin, 2 $\mu\text{g}/\text{mL}$ pepstatin, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethanesulfonyl fluoride, pH 7.4), and sonicated for 10 seconds. SDS sample buffer ($\times 4$) was added to each homogenized sample, and the samples were heated to 100°C for 10 minutes. The samples were then separated by 10% SDS-PAGE (Bio-Rad, Hercules, CA, USA). The resolved proteins were transferred to a nitrocellulose membrane, which was blocked with 5% skim milk in PBS containing 0.1% Tween20 (PBS-T, pH 7.4) for 30 minutes at room temperature. The membrane was then incubated with primary antibodies rabbit anti-NMDAR1 and p-NMDAR1 (1:1 000; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-CaMKII and p-CaMKII (1:1 000; Cell Signaling Technology), rabbit anti-ERK1/2 and p-ERK1/2 (1:1 000; Anti-PhosphoPlus p42/44 MAP kinase Antibody kit, Cell Signaling Technology), rabbit anti-CREB and p-CREB (1:1 000 dilution; PhosphoPlus CREB Antibody kit, Cell Signaling Technology), rabbit anti-GluR1 and p-GluR1 ser831 (1:1 000; Millipore, Billerica, MA, USA), rabbit anti-BDNF (1:1 000; Abcam, Cambridge, UK), and rabbit anti-GDNF (1:1 000; Abcam) in PBS-T overnight at 4°C . After extensive washing with PBS-T and incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:10 000; Thermo Fisher Scientific, Rockford, IL, USA), signals were visualized using a chemiluminescence kit (SuperSignal West Pico; Thermo Fisher Scientific). Membranes were stripped and re-probed with a mouse monoclonal anti- β -actin (1:20 000; Sigma-Aldrich) for normalization. Several exposure times were used to obtain signals in the linear range. The bands were quantified using Scion Image Beta 4.0.2 for Windows XP software (Scion, Frederick, ME, USA).

Immunohistochemistry

Free-floating sagittal sections were cut at a thickness of 30 μm from perfused brains using a sliding microtome (SM2010R; Leica Microsystem, Wetzlar, Germany). The brain from each mouse was sectioned at approximately 1.8 mm laterally, starting from the medial border of hippocampus and extending laterally to the start of the ventral hippocampus. For immunohistochemistry, the sagittal sections were deactivated with endogenous peroxidase (5% hydrogen peroxidase in methyl alcohol and 0.2% Triton X 100) and blocked with 10% normal goat serum (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 hour. Sections were then incubated with primary antibodies, including rabbit anti-p-ERK1/2 (1:500; Cell Signaling Technology), rabbit

anti-p-CREB (1:500 dilution; Cell Signaling Technology), rabbit anti-p-NMDAR1 (1:100; Cell Signaling Technology), rabbit p-CaMKII (1:200; Cell Signaling Technology) and rabbit anti-p-GluR1 ser831 (1:100; Millipore) in PBS-T overnight at 4°C. After three washes, the sections were reacted with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit) for 45 minutes. After three washes, the sections were incubated for 45 minutes with an avidin-biotin peroxidase complex (Vector ABC Elite Kit) prepared according to the manufacturer's instructions. After three washes, the peroxidase reaction was developed for 3 minutes using a diaminobenzidine substrate (DAB kit; 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. The immunohistochemistry-stained specimens were observed using a BX-40 apparatus (Olympus) with a ProgRes® CFscan digital camera (Jenoptik, Jena, Germany).

Statistical analysis

Data are reported as mean \pm SEM and were analyzed using one-way analysis of variance followed by Student-Newman-Keuls *post-hoc* test for multiple comparisons using a GraphPad In Stat (GPIS) computer program (GraphPad Software, San Diego, CA, USA). A *P* value < 0.05 was considered significant.

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Author contributions: Changjong Moon participated in securing funding, study design, technical support, and validated the final version of the manuscript. Miyoung Yang was responsible for data acquisition, integration and analysis, statistical management, and drafting of the manuscript. Juhwan Kim, Sung-Ho Kim, Joong-Sun Kim, and Taekyun Shin participated in the study and provided technical support.

Conflicts of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

Ethical approval: The Institutional Animal Care and Use Committee at Chonnam National University approved the protocols used in this study (CNU IACUC-YB-2009-14).

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