Memantine ameliorates depressive-like behaviors by regulating hippocampal cell proliferation and neuroprotection in olfactory bulbectomized mice

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ABSTRACT

Our previous study suggested that the non-competitive N-methyl-ᴅ-aspartate receptor antagonist memantine (MEM) inhibits dopamine (DA) reuptake and turnover by inhibiting brain monoamine oxidase. Clinical studies have reported that MEM may improve depressive symptoms; however, specific mechanisms underlying this effect are unclear. We performed emotional behavior, tail suspension, and forced swimming tests to examine whether MEM has antidepressant effects in olfactory bulbectomized (OBX) mice, an animal model of depression. Subsequently, we investigated the effects of MEM on the distribution of tyrosine hydroxylase (TH), altered microglia morphometry, and astrocyte and cell proliferation in the hippocampus with immunohistochemistry. We also investigated MEM effects on the levels of norepinephrine (NE), DA, and their metabolites with high performance liquid chromatography, and of neurotrophic, proinflammatory, and apoptotic molecules in the hippocampus with western blotting. Forty-two days after surgery, OBX mice showed depressive-like behaviors, as well as decreased levels of monoamines, reduced cell proliferation, and lower levels of TH, phospho(p)-TH (ser31 and ser40), p-protein kinase A (PKA), p-DARPP-32, p-ERK1/2, p-CREB, brain-derived neurotrophic factor (BDNF), doublecortin, NeuN, and Bcl-2 levels. In contrast, the number of activated microglia and astrocytes and the levels of Iba1, GFAP, p-IκB-α, p-NF-κB p65, TNF-α, IL-6, Bax, and cleaved caspase-3 were increased in the hippocampus. These changes (except for those in NE and Bax) were reversed with chronic administration of MEM. These results suggest that MEM-induced antidepressant effects are associated with enhanced hippocampal cell proliferation and neuroprotection via the PKA-ERK-CREB-BDNF/Bcl-2-caspase-3 pathway and increased DA levels.

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1. Introduction

Olfactory bulbectomized (OBX) mice are a useful experimental animal model for depression, as reported by us and several other researchers. OBX mice express abnormal behaviors, including cognitive deficits and depressive-like and hyperemotional behaviors (Kelly et al., 1997; Hozumi et al., 2003; Takahashi et al., 2011). These abnormal behaviors improve with chronic, but not acute, administration of antidepressant drugs (Breuer et al., 2009a; 2009b). Furthermore, physiological and neurochemical changes in OBX models, such as the reduction of hippocampal monoamines, cell proliferation, and brain-derived neurotrophic factor (BDNF) levels, were similar to those of clinical depression (Nakagawasai et al., 2003a, 2003b; 2016; Takahashi et al., 2016, 2017; Thakare et al., 2017). BDNF is known to play an important role in neuronal survival and is believed to regulate neuroplasticity, including neurogenesis (Waterhouse et al., 2012). Decreased adult hippocampal neurogenesis is associated with depression in both rodents and humans (Scorza et al., 2005; Duman and Monteggia, 2006; Castrén et al., 2007). Moreover, it has been reported that the effects of chronic antidepressant drug administration may be exerted through the enhancement of hippocampal neurogenesis (Santarelli et al., 2003). Thus, in some cases, enhanced neurogenesis in the hippocampus via BDNF signaling pathway activation may mediate the effects of antidepressants.

In contrast, OBX induces neuroinflammation in the hippocampus via activation of microglia, and this inflammation may be associated with depressive-like behavior (Rinwa and Kumar, 2013). The available evidence indicates that depression is closely associated with altered inflammation and microglia activation (Yirmiya et al., 2015), which manifest due to increased inflammatory cytokine levels (Milior et al., 2016). Microglia function is affected by signaling systems associated with depression, namely BDNF (Gomes et al., 2013), glucocorticoids (Ros-Bernal et al., 2011), norepinephrine (NE), or dopamine (DA) (Färber et al., 2005). These reports suggest that microglia-induced inflammation is an important factor in depression. Furthermore, it has been reported that OBX-induced depressive-like behavior is associated with increased inflammatory cytokine and apoptotic factor levels (Rinwa et al., 2013). Antidepressants regulate several apoptotic factors, such as B cell lymphoma/leukemia gene 2 (Bcl-2) expression, which are involved in cell survival pathways (Engel et al., 2013). Thus, neurogenesis and apoptosis may constitute important drug targets for the modulation of depressive symptoms (Lucassen et al., 2006).

Recent clinical and preclinical studies have demonstrated that a subanesthetic single-dose of the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine produces a rapid and sustained antidepressant effect in 70% of treatment-resistant patients with bipolar and major depressive disorder (Berman et al., 2012; Zarate et al., 2006; Diazgranados et al., 2010). Furthermore, our previous study suggested that a non-competitive NMDA receptor antagonist memantine (MEM) inhibits the reuptake and turnover of DA by inhibiting brain monoamine oxidase (MAO) (Onogi et al., 2009). In addition to the already described affinities for the NMDA and D_2 receptors, MEM binds with relatively high affinity to the α7 nicotinic and 5-HT$_3$ receptors (Berman et al., 2012; Kishi and Iwata, 2013; Parsons et al., 2007; Rammes et al., 2001) and possibly interacts with D_1 and 5-HT$_{2A}$ receptors (Ishida et al., 2010; Nakaya et al., 2011). Clinical studies have reported that MEM may improve depressive symptoms, including emotional behavior (Matsunaga et al., 2015; Omranifard et al., 2017); however, the specific detailed mechanisms underlying this effect are unclear.

Therefore, we examined whether MEM improves OBX-induced depressive-like behaviors and investigated the molecular mechanisms underlying this from the perspective of cell proliferation and neuroprotection.

2. Materials and methods

All experiments were performed following the approval of the Ethics Committee of Animal Experiments at Tohoku Medical and Pharmaceutical University, and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize suffering and to reduce the number of animals used. The scores on the behavioral tests were determined manually and measurements were conducted by a blinded observer.

2.1. Animals

We used male ddY strain mice (weighing 28–32 g; Japan SLC, Shizuoka, Japan) for all experiments (total: n = 330; behavioral tests: n = 250; HPLC: n = 16; immunohistochemistry: n = 34; western blot analysis: n = 30). Mice were housed in groups in each cage with free access to food and water under conditions of constant temperature (22 ± 2 °C) and humidity (55 ± 5%), on a 12-h light-dark cycle (lights on: 07:00–19:00).

2.2. Olfactory bulbectomized mice

OBX surgery was performed as previously described (Nakagawasai et al., 2003a). All mice were sacrificed at the end of the experiment and visually examined to confirm that two thirds of the olfactory bulb (OB) had been lesioned. We excluded data from mice in which the lesion was either not extensive enough or extended to the cortex. Sham operations were performed with the same procedure but without the removal of the OB.

2.3. Drugs

MEM (10 and 20 mg/kg; Sigma-Aldrich, St-Louis, USA) was dissolved in physiological saline and chronically intraperitoneally (i.p.) administered once daily between days 14–42 after surgery. Acute treatment of MEM was performed on the 42nd day after surgery.

2.4. Emotional assessments

The evaluation of hyperemotional behaviors in OBX rodents provide a suitable model for evaluating antidepressant efficiency (Saitoh et al., 2003, 2007). At 14–42 days after surgery, emotional behavior was measured with a modified procedure, which has been previously described (Takahashi et al., 2011). All animals underwent emotional behavior observation 30 min after a single drug injection on day 42, or 24 h after the last drug injection after 1, 3, and 4 weeks of administration. Each animal was tested within 10 min.

The study was conducted according to the experimental protocols shown in Fig. 1 (A and B). The emotional behavior of mice was measured by scoring their responses to the following stimuli: attack response, scored by presenting a rod of 4–5 cm in front of the snout; startle response, scored by blowing air on the dorsum using a 5-mL syringe; struggle response, scored by handling with a gloved hand; and fight response, scored by pinching the tail with forceps. The attack response was graded as follows: 0, no reaction; 1, slight; 2, moderate; 3, marked; or 4, extreme reaction. Other responses and vocalization during the test were scored and graded as follows: 0, no reaction or vocalization; 2, moderate; 4, extreme. Fewer gradations were used because it was difficult to discriminate
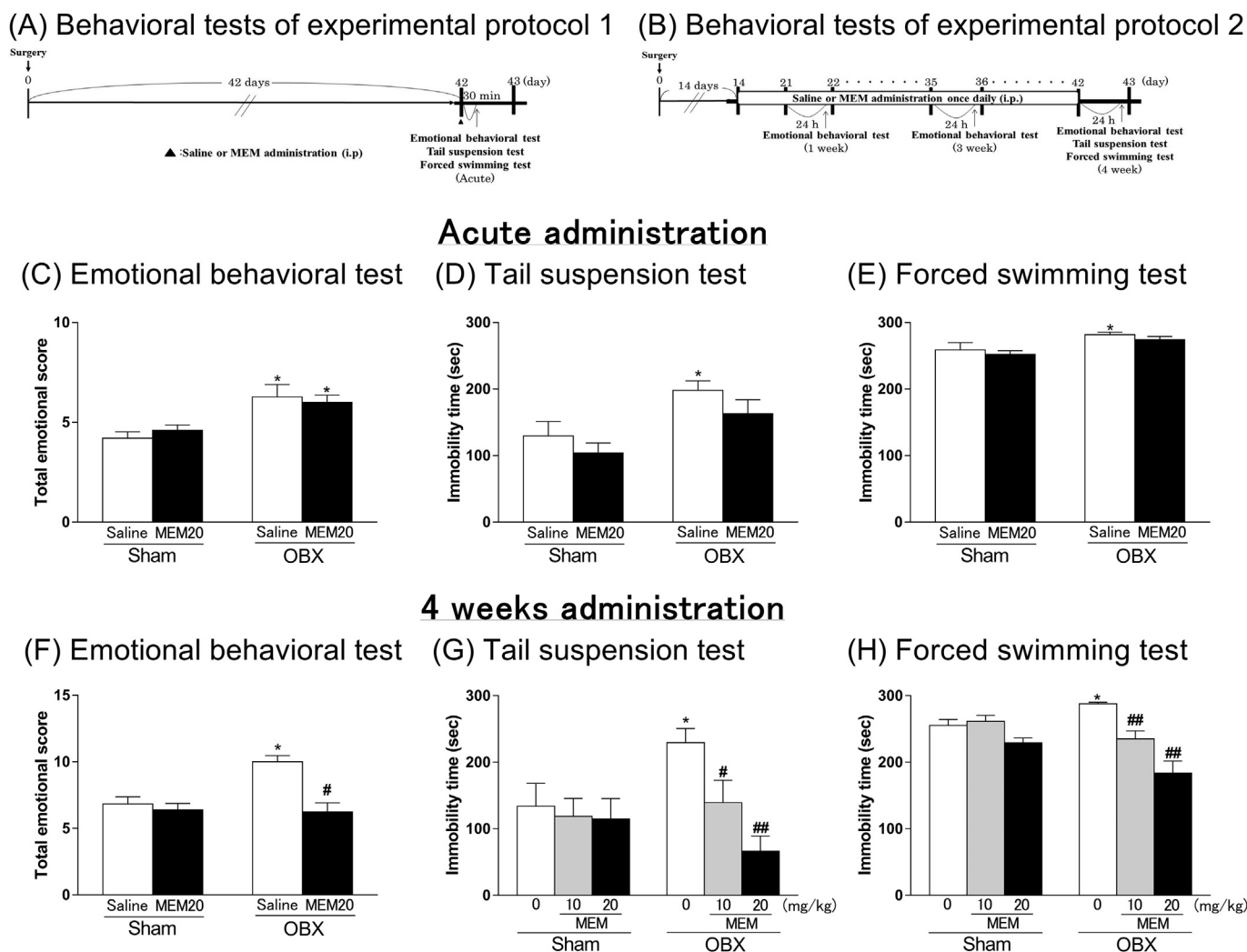
(A) Behavioral tests of experimental protocol 1

(B) Behavioral tests of experimental protocol 2

Acute administration

(C) Emotional behavioral test

(D) Tail suspension test

(E) Forced swimming test

4 weeks administration

(F) Emotional behavioral test

(G) Tail suspension test

(H) Forced swimming test

Fig. 1. Effects of memantine (MEM) on depressive-like behaviors of olfactory bulbectomized (OBX) mice. A, B: Experimental time course for behavioral tests of experimental protocol 1 (A) and of experimental protocol 2 (B). MEM effects were evaluated with an emotional behavior (C and F), tail-suspension (D and G), and forced swim (E and H) tests. The emotional behavior test was conducted after an acute, 1-week, 3-week, and 4-week administration of MEM, while the tail suspension and forced swim tests were conducted only after the acute or 4-week treatment. C–H: Quantification of the total emotional score (C and F) in the emotional test and immobility time in the tail suspension (D and G) and forced swim (E and H) tests. Bars represent means ± SEM. Kruskal—Wallis test: (C), acute administration, $p < 0.01$; (F), chronic administration, $p < 0.01$. Two-way ANOVA [(D), group: $F_{(1, 38)} = 11.88$, $p < 0.01$; treatment: $F_{(1, 38)} = 2.70$, $p > 0.05$; group × treatment: $F_{(1, 38)} = 0.071$, $p > 0.05$; (E), group: $F_{(1, 37)} = 13.29$, $p < 0.01$; treatment: $F_{(1, 37)} = 1.33$, $p > 0.05$; group × treatment: $F_{(1, 37)} = 0.0048$, $p > 0.05$; (G), group: $F_{(1, 50)} = 0.90$, $p > 0.05$; treatment: $F_{(2, 50)} = 4.93$, $p < 0.05$; group × treatment: $F_{(2, 50)} = 3.06$, $p > 0.05$; and (H), group: $F_{(1, 52)} = 2.08$, $p > 0.05$; treatment: $F_{(2, 52)} = 17.79$, $p < 0.01$; group × treatment: $F_{(2, 52)} = 6.66$, $p < 0.01$]. *$p < 0.05$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group ($n = 7$—21 per group).

differences in vocalization. Finally, we evaluated the total emotional score.

2.5. Tail suspension test

The tail suspension test was conducted to assess depressive-like behavior and the effects of antidepressants. Mice were suspended 30 cm above the floor by means of adhesive tape placed approximately 1 cm from the tip of the tail, 30 min after the acute or 24 h after the chronic administration. Mice were considered immobile only when they were hanging completely and were passively motionless. The duration of time spent immobile was quantified during a test period of 10 min.

2.6. Forced swimming test

The forced swimming test was conducted according to a

modified method of Porsolt et al. (1978). As a pre-test, mice were individually placed in vertical glass cylinders (height, 16 cm; diameter, 10 cm) that contained 8 cm of water, maintained at 25 °C, for 15 min. After 24 h, the time during which mice remained immobile was measured during a test period of 5 min. Mice were considered immobile when they passively floated in the water and only made movements necessary to keep their heads above the water line. The forced swimming test was performed 30 min after the acute or 24 h after the chronic drug administration on the 42nd day after surgery.

2.7. Western blotting

Mice were divided into four groups (sham/vehicle, sham/MEM 20 mg/kg, OBX/vehicle, OBX/MEM 20 mg/kg). The mice were sacrificed by decapitation after 4 weeks of saline or MEM administration. Protein isolation and western blots were performed as

described previously (Nakagawasai et al., 2016). After electrophoresis, proteins were transferred to a PVDF membrane, which was then incubated with blocking solution [10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.01% Tween 20, and 5% skim milk] for 1 h and probed with antibodies against tyrosine hydroxylase (TH; 1:100; Millipore Corporation, Billerica, USA), phosphorylated-TH (p-TH, Ser31; 1:1000; Cell Signaling Technology, Danvers, USA), p-TH (Ser40; 1:500; Millipore Corporation), p-protein kinase A (PKA; 1:1000; Cell Signaling Technology), p-DA- and cAMP-regulated phosphoprotein-32 (DARPP-32; 1:1000; Cell Signaling Technology), extracellular signal-regulated protein kinase (ERK; 1:1000; Cell Signaling Technology), p-ERK (1:1000; Cell Signaling Technology), cAMP-responsive element binding protein (CREB; 1:1000; Cell Signaling Technology), p-CREB (1:1000; Cell Signaling Technology), BDNF (1:100; Abcam Ltd., Cambridge, UK), doublecortin (DCX; 1:100; Santa Cruz Biotech, Santa Cruz, CA), neuronal nuclear antigen (NeuN; 1:1000; Millipore Corporation), ionized calcium binding adaptor molecule 1 (Iba1; 1:500; Abcam Ltd.), glial fibrillary acidic protein (GFAP; 1:200; Millipore Corporation), tumor necrosis factor-α (TNF-α; 1:1000; Cell Signaling Technology), interleukin-6 (IL-6; 1:1000; Cell Signaling Technology), p-nuclear factor-kappa B (NF-κB) p65 (1:500; Cell Signaling Technology), p-NF-κB inhibitor-α (IκB-α; 1:1000; Cell Signaling Technology), Bcl-2 (1:200; Santa Cruz Biotechnology), Bcl-2-associated protein X (Bax; 1:1000; Abcam Ltd.), cleaved caspase-3 (1:1000; Cell Signaling Technology), and β-actin (1:1000; Cell Signaling Technology) overnight at 4 °C. The membrane was then washed with blocking solution without milk, incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology/Abcam Ltd.) for 2 h, and the immunoreactive species were visualized with ECL Western blotting detection reagent (Amersham Life Science, Piscataway, USA). The densities of the bands were analyzed with densitometry (Image-J 1.43 μ, National Institute of Health).

2.8. High-performance liquid chromatography (HPLC)

Mice were divided into four groups (sham/saline, sham/MEM 20 mg/kg, OBX/saline, OBX/MEM 20 mg/kg). Sample isolation and measurement of monoamines were performed as described previously (Fukuda et al., 2011). The sample was homogenized in 0.1 M perchloric acid containing 100 ng/mL of isoproterenol as an internal standard. The homogenates were centrifuged at $10,000 \times g$ for 10 min. Supernatants were filtered through a 0.45-μm pore size membrane filter. The filtrate was used for quantification of NE (Sigma-Aldrich), 3-methoxy-4-hydroxymandelic acid (MHA; Sigma-Aldrich), DA (Sigma-Aldrich), dihydroxyphenylacetic acid (DOPAC; Sigma-Aldrich), and homovanillic acid (HVA; Sigma-Aldrich) with HPLC coupled with electrochemical detection. In the mobile phase, we used 95% of 50 mM sodium acetate, 10 mM citric acid, 0.15 mM EDTA, 0.45 mM SOS, and 5% acetonitrile, adjusted to pH 3.6 with glacial acetic acid and filtered to 0.45 μm. The flow rate was 0.4 mL/min. Electrochemical detection was accomplished with an electrochemical detector (model EC8020, Tosoh, Tokyo, Japan) with a glassy working electrode at a potential of 1700 mV. The ratios of MHA/NE, DOPAC/DA, and HVA/DA were used as an index of NE and DA turnover.

2.9. Immunohistochemistry

On day 42 after surgery, 5-bromo-2′-deoxyuridine (BrdU; Sigma–Aldrich; 75 mg/kg) was injected i.p. three times every 2 h after the last administration of saline or MEM. Animals were subsequently sacrificed 24 h after the last injection. Brain samples were collected as described previously (Nakagawasai et al., 2016). The brains were cut into 40 μm sections from bregma −2.20

to −2.80 mm using a cryostat (MICROM HM560, Mikron Instrument, Inc., California, USA).

Frozen sections were mounted on glass slides (Matsunami Glass, Osaka, Japan). Sections were treated with HCl (2 N) at 37 °C for 30 min, followed by neutralization with sodium borate buffer (0.15 M) at room temperature twice every 10 min. After three washes every 5 min, the sections were incubated with phosphate-buffered saline (PBS) containing 1% normal goat serum (Life Technologies Corporation, Carlsbad, USA) and 0.3% Triton X-100 (PBSGT) at room temperature for 2 h. The sections were incubated overnight at 4 °C with rat anti-BrdU (1:100; Harlan SeraLab, Loughborough, UK) and mouse anti-NeuN (1:500; Millipore Corporation) monoclonal antibodies. Sections were washed and incubated for 2 h at room temperature with goat anti-rat IgG Alexa Fluor 568 (1:200; Molecular Probes, Eugene, USA) and goat anti-mouse IgG Alexa Fluor 488 (1:200; Molecular Probes) in PBSGT. Finally, sections were washed and coverslipped with Dako fluorescent mounting medium (Dako, Carpinteria, USA).

Immunofluorescent images were analyzed with a confocal laser-scanning microscope (A1Rsi: Nikon, Tokyo, Japan). Eight sections per mouse were analyzed. We counted the number of BrdU positive cells in both the right and left sides of the dentate gyrus (DG) images (640×640 μm) from each section. The total number of BrdU positive cells was extrapolated for the entire volume of the DG.

2.10. Neuromorphometrical study

The brain samples were collected as described previously (Nakagawasai et al., 2016) and in paragraph 2.9. The sections were incubated overnight at 4 °C with rabbit anti-TH (1:100; Millipore Corporation), rabbit anti-Iba1 (1:200; Wako Pure Chemical Industries Ltd, Osaka, Japan), and mouse GFAP (1:200; Millipore Corporation) antibodies. Sections were washed and incubated for 2 h at room temperature with goat anti-rabbit IgG Alexa Fluor 568 (1:200; Molecular Probes) and goat anti-mouse IgG Alexa Fluor 488 (1:200; Molecular Probes) in PBSGT. We observed alterations in TH-positive cells in OBX mice with a confocal laser-scanning microscope. We then evaluated the activation of microglia and astrocytes by observing and counting cells that typically exhibited hypertrophy with thicker processes, and larger and densely stained cell bodies.

2.11. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The significance of differences was determined by a two-way analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) test for multigroup comparisons. Regarding the emotional behavioral test, the statistical significance of differences was assessed with a non-parametric Kruskal–Wallis test followed by Steel's test (non-parametric statistical analysis). We considered $p < 0.05$ for representing significant differences.

3. Results

3.1. Effects of MEM on OBX-induced depressive-like behaviors

We measured emotional behavior to clarify whether emotional responses in OBX mice were affected by an acute, 1-week, 3-week, or 4-week treatment with MEM (20 mg/kg). There was a significant increase in the total emotional score in the OBX + saline group on day 42 after surgery compared with the sham + saline group [Fig. 1 (C-F)]. This was reversed after the 4-week chronic administration of MEM (20 mg/kg) [Fig. 1 (F)], but not after the acute administration
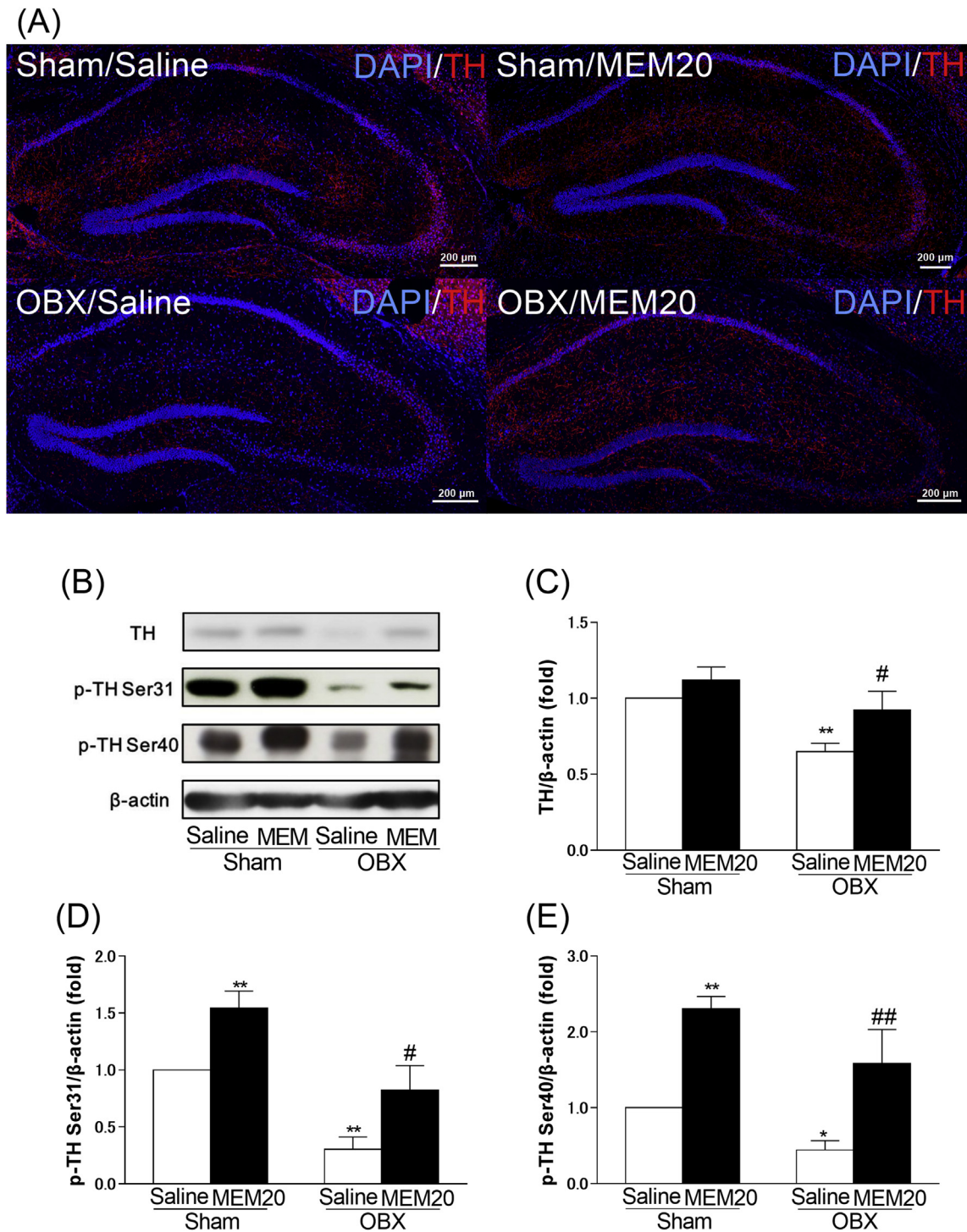
Fig. 2. Effects of memantine (MEM) on the levels of total and activated tyrosine hydroxylase (TH). A: Microscopy images of TH (red) and DAPI (blue) immunostaining in the hippocampus. Images show alterations in TH, p-TH^{Ser31}, and p-TH^{Ser40} levels in the hippocampus after MEM administration. B: Representative immunoblots probed with antibodies against TH, p-TH^{Ser31}, p-TH^{Ser40}, and β-actin. C—E: Quantification of the normalized to β-actin levels of TH, p-TH^{Ser31}, and p-TH^{Ser40} after saline or MEM treatment in sham-operated or olfactory bulbectomized (OBX) mice. Bars represent means ± SEM. Two-way ANOVA [(C), group: $F_{(1, 12)} = 11.72$, $p < 0.01$; treatment: $F_{(1, 12)} = 6.05$, $p < 0.05$; group × treatment: $F_{(1, 12)} = 0.9$, $p > 0.05$; (D), group: $F_{(1, 12)} = 34.14$, $p < 0.01$; treatment: $F_{(1, 12)} = 19.28$, $p < 0.01$; group × treatment: $F_{(1, 12)} = 0.011$, $p > 0.05$; and (E), group: $F_{(1, 12)} = 6.83$, $p < 0.05$; treatment: $F_{(1, 12)} = 25.23$, $p < 0.01$; group × treatment: $F_{(1, 12)} = 0.11$, $p > 0.01$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group (n = 3—5 per group).

[Fig. 1 (C)]. On days 21 (1 week of drug treatment) and 35 (3 weeks of drug treatment) after surgery, we observed no differences between the OBX + saline and OBX + MEM groups (data not shown). Furthermore, on day 42 after surgery, OBX control mice remained immobile for a significantly longer period than sham controls in both the tail suspension and forced swimming tests. In contrast, OBX mice, chronically treated with MEM (10 and 20 mg/kg), remained immobile for significantly shorter periods than the OBX

controls in both tests [Fig. 1 (G and H)], whereas acute administration of MEM (20 mg/kg) did not have this effect [Fig. 1 (D and E)]. In contrast, there was no change in the duration of immobility in either test between sham mice treated with MEM (10 and 20 mg/kg) and sham controls. These results suggest that chronic MEM-treatment improves OBX-induced depressive-like behaviors.

3.2. Changes in TH and monoamine levels in the hippocampus of OBX mice

Our previous study demonstrated that OBX induces a decrease in striatal TH levels, a biosynthetic rate-controlling enzyme of catecholamines, such as NE and DA (Takahashi et al., 2016). Thakare et al. (2017) demonstrated a decrease in monoamines in the hippocampus of OBX mice. As the pathophysiology of depression is associated with low levels of monoamines in the central nervous system, like NE and DA, (Carlsson et al., 1969; Schildkraut and Kety, 1967), we examined whether OBX can decrease TH levels and activity in the hippocampus.

As shown in Fig. 2, the TH, p-TH^{ser31}, and p-TH^{ser40} immunocontent was significantly lower in OBX mice than in sham controls. Compared with saline, MEM treatment significantly increased TH levels in the hippocampus of OBX mice, and significantly increased p-TH^{ser31} and p-TH^{ser40} levels in the hippocampus of both groups.
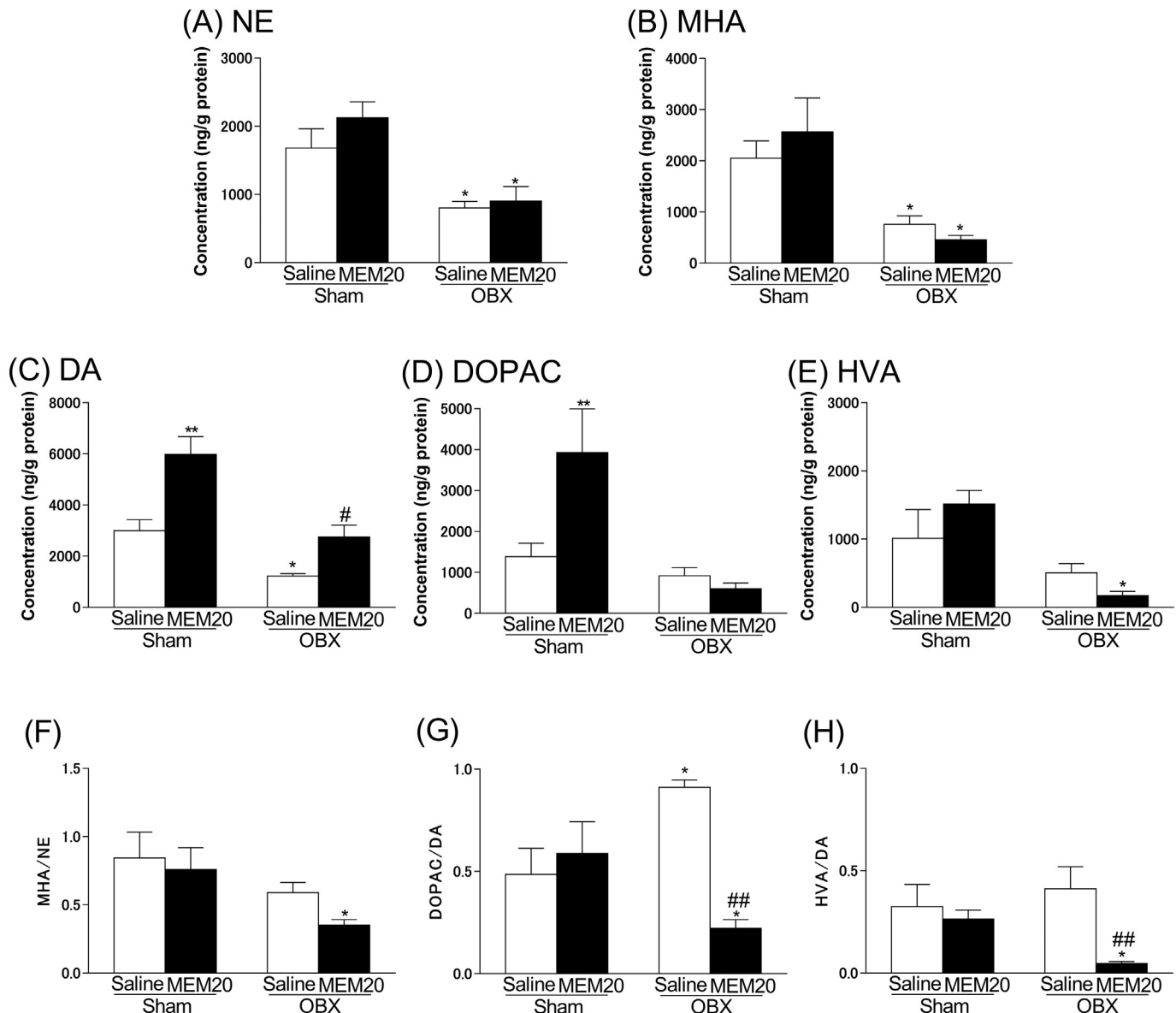

Fig. 3. Altered norepinephrine (NE), MHA (3-methoxy-4-hydroxymandelic acid), dopamine (DA), DOPAC (dihydroxyphenylacetic acid), and HVA (homovanillic acid) concentrations and turnover in the hippocampus of olfactory bulbectomized (OBX) mice. A—E: Graphs showing the quantification of the concentrations of NE (A), MHA (B), DA (C), DOPAC (D), and HVA (E) in the hippocampus of sham or OBX mice treated with saline or MEM. Concentrations are expressed as ng per g weight of fresh brain tissue. Bars represent means ± SEM. Two-way ANOVA [(A), group: $F_{(1, 12)} = 23.37$, $p < 0.01$; treatment: $F_{(1, 12)} = 1.56$, $p > 0.05$; group × treatment: $F_{(1, 12)} = 0.62$, $p > 0.05$; (B), group: $F_{(1, 12)} = 19.9$, $p < 0.01$; treatment: $F_{(1, 12)} = 0.076$, $p > 0.05$; group × treatment: $F_{(1, 12)} = 1.16$, $p > 0.05$; (C), group: $F_{(1, 12)} = 28.47$, $p < 0.01$; treatment: $F_{(1, 12)} = 23.1$, $p < 0.01$; group × treatment: $F_{(1, 12)} = 2.41$, $p > 0.05$; (D), group: $F_{(1, 12)} = 11.04$, $p < 0.01$; treatment: $F_{(1, 12)} = 3.8$, $p > 0.05$; group × treatment: $F_{(1, 12)} = 6.29$, $p < 0.05$; and (E), group: $F_{(1, 12)} = 14.04$, $p < 0.01$; treatment: $F_{(1, 12)} = 0.12$, $p > 0.05$; group × treatment: $F_{(1, 12)} = 2.87$, $p > 0.05$]. F—H: Graphs showing the MHA/NE (F), DOPAC/DA (G), and HVA/DA (H) ratios. Bars represent means ± SEM. Two-way ANOVA [(F), group: $F_{(1, 12)} = 6.28$, $p < 0.05$; treatment: $F_{(1, 12)} = 1.50$, $p > 0.05$; group × treatment: $F_{(1, 12)} = 0.34$, $p > 0.05$; (G), group: $F_{(1, 12)} = 0.08$, $p > 0.05$; treatment: $F_{(1, 12)} = 7.92$, $p < 0.05$; group × treatment: $F_{(1, 12)} = 14.42$, $p < 0.01$; and (H), group: $F_{(1, 12)} = 0.43$, $p > 0.05$; treatment: $F_{(1, 12)} = 7.19$, $p < 0.05$; group × treatment: $F_{(1, 12)} = 3.70$, $p > 0.05$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group (n = 4 per group).

From these results, we hypothesized that monoamines were decreased in the hippocampus of OBX mice, and that this could be reversed by MEM.

Next, we measured the levels of NE, DA, and their metabolites in the hippocampus of OBX mice [Fig. 3 (A—E)]. Sham mice treated with MEM had significantly higher levels of DA and DOPAC than the sham control group. We observed significantly lower NE, MHA, and DA concentrations in the OBX than the sham control group, while OBX mice treated with MEM had significantly higher DA levels. Moreover, we observed that the DOPAC/DA ratio was significantly higher in OBX mice than in sham controls, while the ratios of DOPAC/DA and HVA/DA were reduced after the administration of MEM in OBX mice [Fig. 3 (G and H)]. These results suggest that the antidepressant effects of MEM may underlie, at least in part, the increase in DA concentration in the hippocampus, via the inhibition of DA turnover.

3.3. Changes in p-PKA, p-DARPP-32, p-ERK, p-CREB, and BDNF levels in the hippocampus of OBX mice

In the present study, MEM led to increased DA levels in the hippocampus of OBX mice. We examined whether the magnitude of this increase is functionally meaningful. The stimulation of DA receptors activates PKA, DARPP-32, and the ERK-CREB-BDNF pathway (Bozzi et al., 2011). As shown in Fig. 4, the levels of p-PKA, p-DARPP-32, p-ERK, p-CREB, and BDNF were significantly lower in OBX mice than in sham controls. Compared with saline, MEM treatment significantly increased p-PKA and p-DARPP-32

levels in the hippocampal DG of both the sham and OBX groups. Similarly, MEM treatment increased p-ERK, p-CREB, and BDNF levels in the hippocampus of OBX mice. These results suggest that increased DA, induced by MEM, is functionally meaningful and that MEM-induced antidepressant effects might underlie the increased BDNF levels in the hippocampus, via the PKA-ERK-CREB pathway, by increasing DA.

3.4. Effect of MEM on reduced cell proliferation in the hippocampal DG of OBX mice

In the present study, MEM enhanced BDNF levels, which regulate forms of plasticity including neurogenesis in the hippocampus (Waterhouse et al., 2012). Therefore, animals were injected with BrdU on the 42nd day after surgery to determine the rate of hippocampal cell proliferation. Anti-NeuN antibody was used to identify mature neurons in the DG area. The incorporation of BrdU into a cell indicates that it was cycling at the time of the BrdU injection. We found a significantly lower number of BrdU positive cells in OBX than in sham mice [Fig. 5 (A and B)]. However, the chronic administration of MEM (20 mg/kg) significantly increased the number of BrdU positive cells in OBX mice.

In order to examine whether the excess of newborn cells differentiates into immature and mature neurons in the hippocampus, we investigated changes in DCX and NeuN, markers of immature and mature neurons, respectively, after MEM treatment. As shown in Fig. 5 (C—E), the immunocontents of DCX and NeuN in OBX mice were significantly lower than in sham controls. Compared with
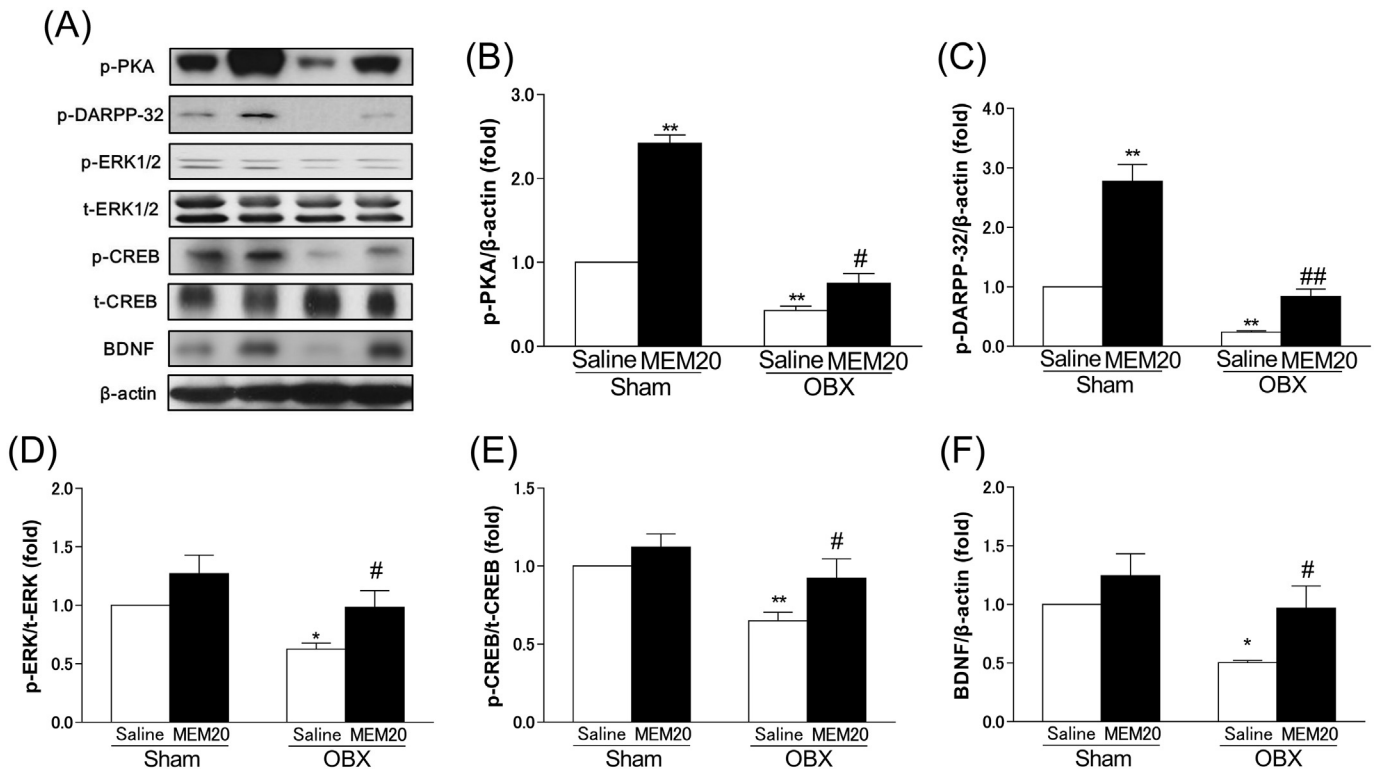

Fig. 4. Altered levels of the phosphorylated (p) forms of PKA, DARPP-32, ERK, and CREB, and of BDNF in the hippocampus after memantine (MEM) administration. A: Representative immunoblots probed with antibodies against p-PKA, p-DARPP-32, p-ERK, t-ERK, p-CREB, t-CREB, BDNF, and β-actin, as indicated. B—F: Quantification of the normalized values of p-PKA (B), p-DARPP-32 (C), and BDNF (F) levels with β-actin and of p-ERK (D) and p-CREB (E) with total (t)-ERK and t-CREB, respectively. Bars represent means ± SEM. Two-way ANOVA [(B), group: $F_{(1, 9)} = 227.5$, $p < 0.01$; treatment: $F_{(1, 9)} = 137.3$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 54.15$, $p < 0.01$; (C), group: $F_{(1, 12)} = 125.8$, $p < 0.01$; treatment: $F_{(1, 12)} = 96.87$, $p < 0.01$; group × treatment: $F_{(1, 12)} = 23.98$, $p < 0.01$; (D), group: $F_{(1, 14)} = 11.54$, $p < 0.01$; treatment: $F_{(1, 14)} = 10.51$, $p < 0.01$; group × treatment: $F_{(1, 14)} = 0.20$, $p > 0.05$; (E), group: $F_{(1, 12)} = 11.72$, $p < 0.01$; treatment: $F_{(1, 12)} = 6.05$, $p < 0.05$; group × treatment: $F_{(1, 12)} = 0.90$, $p > 0.05$; and (F), group: $F_{(1, 15)} = 9.19$, $p < 0.01$; treatment: $F_{(1, 15)} = 7.67$, $p < 0.05$; group × treatment: $F_{(1, 15)} = 0.73$, $p > 0.05$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group (n = 3—5 per group).
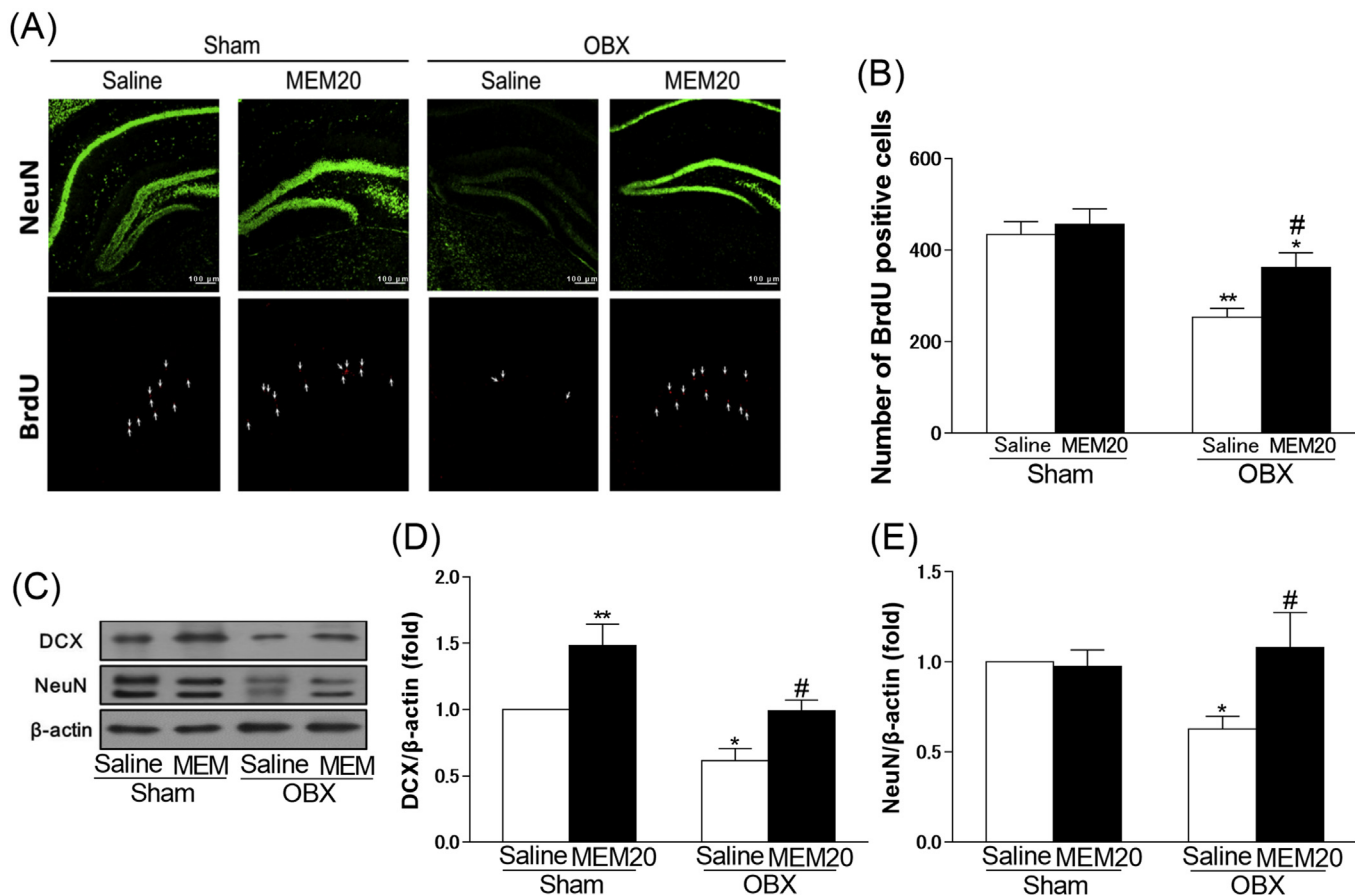
Fig. 5. Influence of memantine (MEM) on hippocampal neurogenesis in olfactory bulbectomized (OBX) mice. A: Microscopy images showing BrdU (red) and NeuN (green) immunostaining in the dentate gyrus region of the hippocampus. Arrows indicate BrdU-labeled cells. B: Quantitative analysis of the number of BrdU positive cells in sham and OBX mice treated with saline or MEM. Bars represent means ± SEM. Two-way ANOVA [(B), group: $F_{(1, 16)} = 47.59$, $p < 0.01$; treatment: $F_{(1, 16)} = 13.15$, $p < 0.01$; group × treatment: $F_{(1, 16)} = 0.16$, $p > 0.05$]. C: Representative immunoblots probed with antibodies against DCX, NeuN, and β-actin, as indicated. Altered DCX and NeuN levels in the hippocampus were observed after MEM administration. D, E: Quantification of normalized values of DCX and NeuN levels with β-actin. Bars represent means ± SEM. Two-way ANOVA [(D), group: $F_{(1, 18)} = 16.85$, $p < 0.01$; treatment: $F_{(1, 18)} = 17.5$, $p < 0.01$; group × treatment: $F_{(1, 18)} = 0.27$, $p > 0.05$; and (E), group: $F_{(1, 17)} = 3.84$, $p > 0.05$; treatment: $F_{(1, 17)} = 1.53$, $p > 0.05$; group × treatment: $F_{(1, 17)} = 4.83$, $p < 0.05$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ vs. saline-treated OBX group (n = 5–6 per group).

saline, MEM treatment significantly decreased DCX and NeuN levels in the hippocampus of OBX mice. In contrast, DCX but not NeuN levels in the hippocampus were significantly higher in sham mice treated with MEM. We suggest that MEM-induced antidepressant effects in OBX mice may be associated with enhanced cell proliferation in the DG and the enhancement of BDNF levels.

3.5. Effect of MEM on the enhancement of inflammation in the hippocampus of OBX mice

Acute and chronic neuroinflammation can negatively affect many stages of neurogenesis in the adult mammalian brain, including proliferation, differentiation, and survival of newborn neurons (Hashimoto, 2015; Miller et al., 2009). To examine whether OBX induces the activation of microglia and astrocytes in the hippocampus, we performed immunostaining and western blotting by using antibodies for Iba1 and GFAP [Fig. 6 (A–D)]. Compared with sham controls, the microglia marker Iba1 and the astrocyte marker GFAP were significantly increased in the hippocampus of OBX mice. We observed that activated microglia and astrocytes typically exhibited hypertrophy, with thicker processes, larger and more densely stained cell bodies, while the number of activated glial cells was increased in the hippocampus of OBX mice. These phenomena were also observed in the DG area of OBX mice. In contrast,

activation of microglia and astrocytes in the hippocampus of MEM-treated OBX mice was attenuated. As shown in Fig. 6 (C and D), Iba1 and GFAP immunocontent was significantly higher in OBX mice than in sham controls. Compared with saline, MEM treatment significantly decreased Iba1 and GFAP levels in the hippocampus of OBX mice. Moreover, MEM reversed the effects of OBX on glial cell activation, including on cell body size, processes, cell count, and cell density [Fig. 6 (I-P)]. These results suggest that OBX induces neuroinflammation in the hippocampus via activation of microglia and astrocytes, while MEM appears to have an anti-neuroinflammatory effect.

3.6. Changes in p-IκB-α, p-NF-κB p65, TNF-α, and IL-6 levels in the hippocampus of OBX mice

Microglia are important resident immunoreactive cells in the central nervous system. The available evidence indicates that depression is intimately associated with altered inflammation and microglia (Yirmiya et al., 2015), manifesting with an increased proinflammatory profile, involving increased TNF-α and IL-6 levels (Milior et al., 2016). Thus, we examined whether OBX-induced neuromorphometrical alterations of microglia and astrocytes are functionally meaningful.

As shown in Fig. 7, the immunocontent of p-IκB-α, p-NF-κB p65,

TNF-α, and IL-6 was significantly higher in OBX mice than in sham controls. Compared with saline, MEM treatment significantly decreased p-IκB-α, p-NF-κB p65, TNF-α, and IL-6 levels in the hippocampus of OBX mice. Moreover, OBX-induced neuroinflammation, leading to increased TNF-α and IL-6 levels via the activation of p-IκB-α and p-NF-κB p65, was reversed by MEM. These results suggest that OBX-induced neuromorphometrical alterations of microglia and astrocytes are functionally meaningful.

3.7. Changes in Bcl-2, Bax, and cleaved caspase-3 levels in the hippocampus of OBX mice

Modulation of apoptosis may play an important role in the modulation of depressive symptoms (Lucassen et al., 2006). Since the balance between Bcl-2 and Bax is involved in the regulation of apoptotic cell death (Cory and Adams, 2002) and cleaved caspase-3 is a crucial mediator of apoptosis (Fiandalo and Kyprianou, 2012), we next investigated whether OBX influences Bcl-2, Bax, and cleaved caspase-3 expression.

As shown in Fig. 8 (A), the Bcl-2 immunocontent was significantly lower in OBX than in sham mice. Compared with saline, MEM treatment significantly increased Bcl-2 levels in the hippocampus of OBX mice. In contrast, the Bax immunocontent was significantly higher in OBX mice treated with either saline or MEM than in sham controls [Fig. 8 (B)]. Similarly, cleaved caspase-3 levels were significantly higher in OBX than in sham mice, and this change was reversed by MEM. In contrast, cleaved caspase-3 immunocontent significantly increased in sham mice treated with MEM compared to sham controls [Fig. 8 (C)]. These results demonstrate that neuronal apoptosis is induced by OBX and that treatment with MEM significantly reverses this effect.
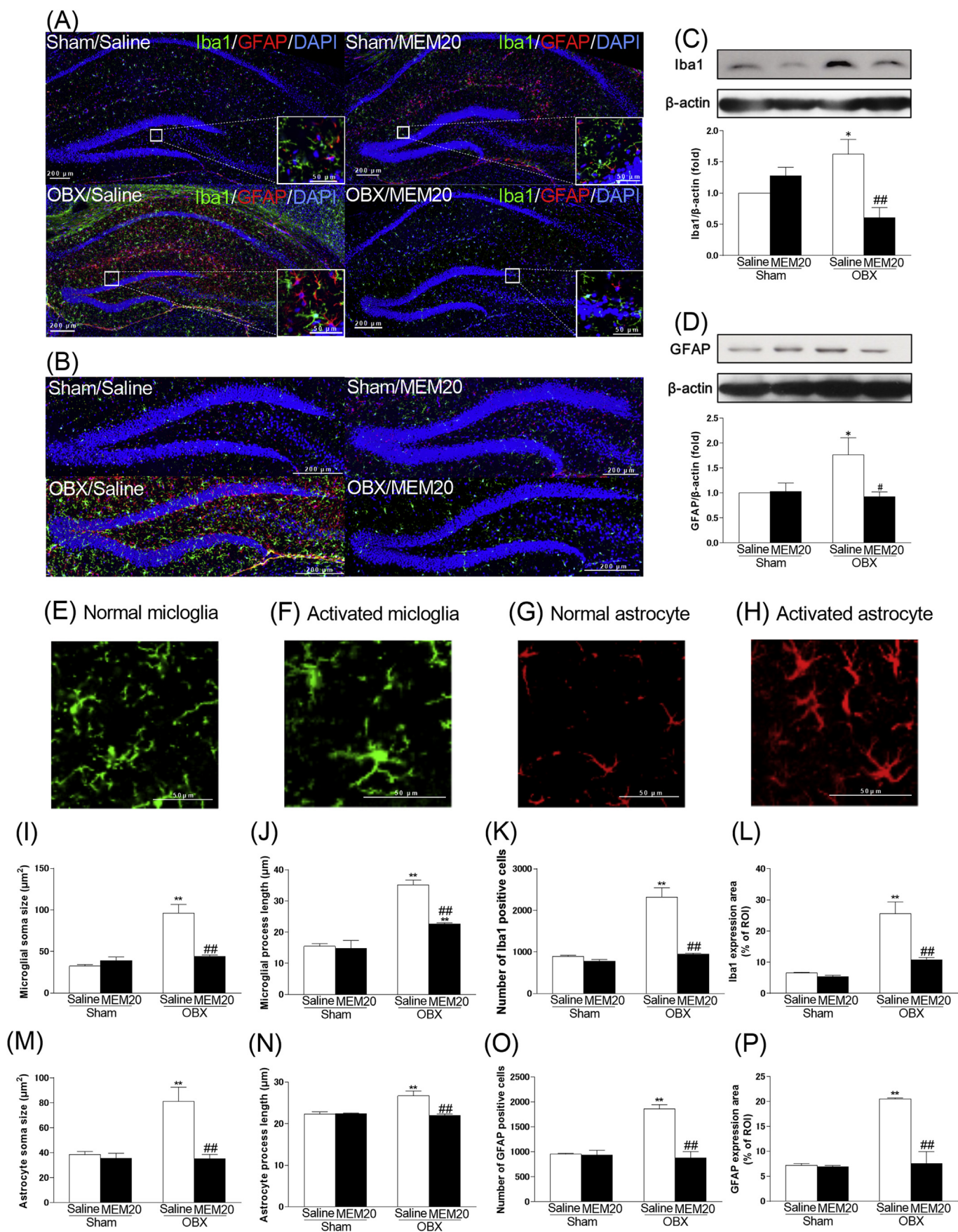
4. Discussion

The present study showed that OBX mice exhibit depressive-like behaviors in the tail suspension, forced swimming, and emotional behavior tests, 42 days after surgery. Furthermore, chronic, but not acute, administration of MEM improved OBX-induced depressive-like behavior. We observed that MEM (10 or 20 mg/kg, i.p.) has antidepressant effects when administered chronically for 4 weeks after OBX surgery in mice. In contrast, Borre et al. reported that MEM [20 mg/kg, per os (p.o.)] has antidepressant effects when administered before the OBX surgery in rats (Borre et al., 2012). We propose that the administration method, species differences, or evaluation of specific behavioral aspects may be responsible for these discrepancies. For example, drugs reach the brain more efficiently with i.p. than p.o. administration, suggesting that the concentration of MEM in the brain examined in our study may be higher than that examined in the study by Borre et al. Moreover, it has been reported that OBX in different species occasionally exhibits different outcomes (Hendriksen et al., 2015). In the striatum, an area of the dopaminergic system associated with motivation and anhedonia in depression patients, OBX mice exhibit decreased TH levels (Takahashi et al., 2016). On the other hand, OBX rats exhibit increased DA levels in this area (Masini et al., 2004). Moreover, OBX mice but not OBX rats exhibit depressive-like behavior, including longer immobility time during the forced swimming test (Han et al., 2009; Kelly and Leonard, 1999). Regarding the behavioral aspects, Borre et al. evaluated the effects of MEM on OBX-induced hyperactivity, which may reflect psychomotor retardation or agitation. In contrast, our study evaluated MEM effects on OBX-induced hyperemotional response and immobility duration, which may reflect mood, motivation, or remissness (Abelaira et al., 2013). These factors may be responsible for the different results.

Clinical and preclinical studies have reported that MEM ameliorates depressive-like symptoms, including emotional behavior (Matsunaga et al., 2015; Omranifard et al., 2017; Kos and Popik, 2005). Other studies have reported that the antidepressant effects of MEM are associated with the 5-HT₃ receptor and antagonism of NMDA receptors in the central nervous system (Rammes et al., 2001; Réus et al., 2010). However, we previously reported that MEM inhibits DA reuptake and turnover by inhibiting brain MAO activity (Onogi et al., 2009). Thus, in this study, we focused on the role of the dopaminergic system in the antidepressant effects of MEM.

Consistent with our results, previous studies have suggested that NE and DA levels are decreased in the hippocampus of OBX mice (Thakare et al., 2017). These changes might be, at least in part, attributed to the decreased TH levels and activity in the hippocampus of these mice. The pathophysiology of depression involves decreased 5-HT, NE, and DA levels in the central nervous system (Carlsson et al., 1969; Schildkraut and Kety, 1967). Clinical studies have reported that monoamine replacement is an important factor towards the effective treatment of patients with depression (Carroll, 1971). In the present study, MEM treatment significantly enhanced TH activity and increased DA concentration in the hippocampus of mice in both the sham and OBX groups. These effects have also been demonstrated in studies using primary midbrain neuron-glial cultures, in which MEM was shown to increase TH-positive cells and DA levels (Wu et al., 2009). Preclinical and clinical studies have reported that increased DA levels in the brain show antidepressant effects (Hosenbocus and Chahal, 2013; Chen et al., 2016). We also observed a significant increase in the DOPAC/DA ratio of OBX mice compared with the one in sham controls. Previously, we demonstrated that OBX increases MAO_B activity, which leads to DA metabolism in the forebrain (Nakagawasai et al., 2003b). Thus, we suggest that the increased DOPAC/DA ratio in OBX mice might be induced by activation of MAO_B. Moreover, DA turnover (DOPAC/DA and HVA/DA) was inhibited in OBX mice treated with MEM. This might be explained by our previous study in which we showed that MEM inhibits the reuptake of DA and MAO_B in the forebrain (Onogi et al., 2009). Taken together, these results suggest that DA is involved in the modulation of MEM-induced antidepressant effects.

Takamura et al. (2014) suggested that DA might play a role in adult hippocampal neurogenesis via DA receptors, and that activation of these receptors holds a therapeutic potential for depression. We observed that the low levels of p-PKA, p-DARPP-32, p-ERK1/2, p-CREB, and BDNF in the hippocampus of OBX mice were reversed after chronic treatment with MEM. Phosphorylation of DARPP-32 plays an important role in mediating dopaminergic transmission (Walaas et al., 1983). Furthermore, the state of phosphorylation of this protein in dopaminergic neurons is regulated by both DA and cAMP. Thus, we suggest that increased DA levels, induced by MEM, stimulate DA receptors. Moreover, enhanced DA activates the PKA and ERK1/2 pathways (Bozzi et al., 2011). In turn, these pathways lead to the activation of CREB, important for neural plasticity, as well as to the transcription of BDNF (Finkbeiner et al., 1997) and TH genes (Gueorguiev et al., 2006). Results from several studies have led to the formulation of the neurotrophic hypothesis of depression, based on which low levels of BDNF lead to a depressive condition (Castrén et al., 2007; Duman and Monteggia, 2006). Neurotrophins are trophic proteins essential for neuronal survival and differentiation, while high hippocampal BDNF levels attenuate depression symptoms (Chen et al., 2001). Moreover, ERK1/2 pathway promotes the activation of TH by increasing its phosphorylation on ser31 (Haycock et al., 1992), while PKA induces TH phosphorylation on ser40 (Funakoshi et al., 1991). The present study revealed that MEM significantly increases p-PKA, p-ERK1/2, p-TH^ser31, and p-TH^ser40 levels in both the sham and OBX groups (p-
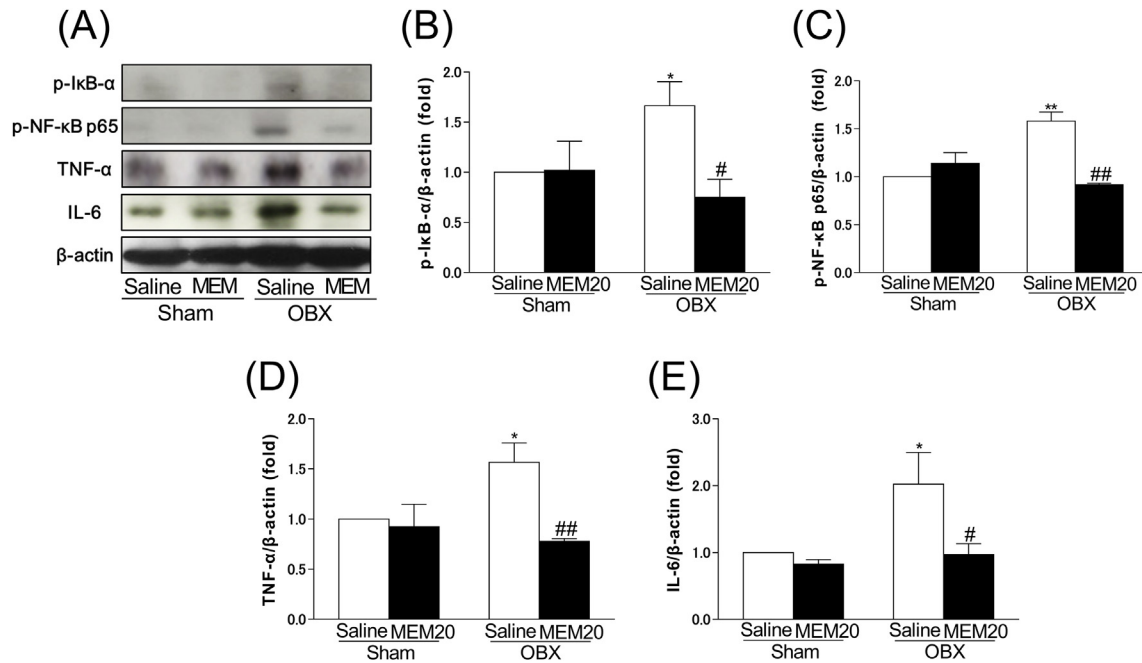
Fig. 7. Altered levels of phosphorylated (p)-IκBα, p-NF-κB p65, TNF-α, and IL-6 in the hippocampus after memantine (MEM) administration. A: Representative immunoblots probed with antibodies against p-IκBα, p-NF-κB p65, TNF-α, IL-6, and β-actin, as indicated. B–E: Quantification of normalized values of p-IκBα, p-NF-κB p65, TNF-α, and IL-6 levels with β-actin. Bars represent means ± SEM. Two-way ANOVA [(B), group: $F_{(1, 15)} = 0.86$, $p > 0.05$; treatment: $F_{(1, 15)} = 4.36$, $p > 0.05$; group X treatment: $F_{(1, 15)} = 4.73$, $p < 0.05$; (C), group: $F_{(1, 14)} = 6.11$, $p < 0.05$; treatment: $F_{(1, 14)} = 12.96$, $p < 0.01$; group X treatment: $F_{(1, 14)} = 30.75$, $p < 0.01$; (D), and group: $F_{(1, 14)} = 2.01$, $p > 0.05$; treatment: $F_{(1, 14)} = 8.55$, $p < 0.05$; group X treatment: $F_{(1, 14)} = 5.85$, $p < 0.05$; and (E), group: $F_{(1, 15)} = 4.83$, $p < 0.05$; treatment: $F_{(1, 15)} = 5.35$, $p < 0.05$; group X treatment: $F_{(1, 15)} = 2.73$, $p > 0.05$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group (n = 3–5 per group).

ERK1/2 levels in the sham group were slightly increased (p = 0.08) by MEM compared to saline). Thus, our results suggest that MEM-induced antidepressant effects might underlie the increased BDNF levels in the hippocampus and that MEM-induced enhancement of TH levels and activity in the hippocampus might be associated with PKA-ERK-CREB pathway via increasing DA.

It is well known that adult neurogenesis occurs mainly in two regions of the brain, one of which is the subgranular zone of the DG in the hippocampal formation (Alvarez-Buylla and Garcia-Verdugo, 2002; Cayre et al., 2002). In the present study, OBX mice showed a significant decrease in cell proliferation in the DG, consistently with our previous study (Nakagawasai et al., 2016; Takahashi et al., 2017). Moreover, DCX and NeuN levels in the hippocampus were significantly lower in OBX than in sham mice. Likewise, DeCarolis and Eisch (2010) reported a reduction in neurogenesis in the hippocampus of patients with depression. Moreover, Santarelli et al. (2003) suggested that antidepressant effects may be mediated by the enhancement of neurogenesis in the hippocampus. These findings suggest that enhancement of neurogenesis in the hippocampus may ameliorate depressive-like behaviors in the OBX

model. We observed that chronic administration of MEM to OBX mice significantly increases the number of newborn cells in the hippocampus. Thus, we suggest that MEM-induced antidepressant effects might be mediated by the induction of neurogenesis in the hippocampal DG of OBX mice.

Neuroinflammation can negatively affect many stages of neurogenesis in the adult mammalian brain, including proliferation, differentiation, and survival of newborn neurons (Hashimoto, 2015; Miller et al., 2009). Current evidence points to a role of inflammatory processes in the pathophysiology of depression (Hashimoto, 2009, 2015; Miller et al., 2009). Patients with depression show increased blood concentrations of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Howren et al., 2009; Kim et al., 2007). Moreover, antidepressant treatment attenuates the expression of inflammatory biomarkers in depression (Maes et al., 2009), while depression is intimately associated with altered inflammation and microglia (Yirmiya et al., 2015). Indeed, results from many studies converge to suggest that microglia are morphologically altered in frontal limbic regions of patients with depression and suicide completers (Steiner et al., 2008; Schnieder et al., 2014;

Fig. 6. Effects of memantine (MEM) on microglia and astrocyte activation in the hippocampus of olfactory bulbectomized (OBX) mice. A, B: Microscopy images of Iba1 (green), GFAP (red), and DAPI (blue) immunostaining in the hippocampus (A) and dentate gyrus (B). Altered Iba1 and GFAP levels in the hippocampus were observed after MEM administration. C, D: Representative immunoblots probed with antibodies against Iba1 (C), GFAP (D), and β-actin are shown. Graphs in panels C and D indicate the quantification of the normalized values of Iba1 and GFAP levels with β-actin. Bars represent means ± SEM. Two-way ANOVA [(C), group: $F_{(1, 14)} = 0.016$, $p > 0.05$; treatment: $F_{(1, 14)} = 5.04$, $p < 0.05$; group × treatment: $F_{(1, 14)} = 15.3$, $p < 0.01$; (D), group: $F_{(1, 13)} = 2.80$, $p > 0.05$; treatment: $F_{(1, 13)} = 4.24$, $p > 0.05$; group × treatment: $F_{(1, 13)} = 4.98$, $p < 0.05$]. E–H: Microscopy images showing normal and activated microglia (E and F) and astrocytes (G and H), as indicated. I–P: Quantification of soma size (I and M), process length (J and N), Iba1 positive cells (K), GFAP positive cells (O), and cell density in the region of interest (ROI), using representative tissue sections stained with Iba1 and GFAP antibodies. Bars represent means ± SEM. Two-way ANOVA [(I), group: $F_{(1, 9)} = 38.93$, $p < 0.01$; $F_{(1, 9)} = 17.47$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 28.98$, $p < 0.01$; (J), group: $F_{(1, 9)} = 92.22$, $p < 0.01$; treatment: $F_{(1, 9)} = 21.12$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 16.97$, $p < 0.01$; (K), group: $F_{(1, 9)} = 55.9$, $p < 0.01$; treatment: $F_{(1, 9)} = 44.7$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 34.75$, $p < 0.01$; (L), group: $F_{(1, 9)} = 45.58$, $p < 0.01$; treatment: $F_{(1, 9)} = 19.57$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 14.4$, $p < 0.01$; (M), group: $F_{(1, 9)} = 10.01$, $p < 0.01$; treatment: $F_{(1, 9)} = 13.67$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 14.66$, $p < 0.01$; (N), group: $F_{(1, 9)} = 11.93$, $p < 0.01$; treatment: $F_{(1, 9)} = 15.95$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 12.66$, $p < 0.01$; (O), group: $F_{(1, 9)} = 17.19$, $p < 0.01$; treatment: $F_{(1, 9)} = 23.79$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 21.93$, $p < 0.01$; and (P), group: $F_{(1, 9)} = 20.4$, $p < 0.01$; treatment: $F_{(1, 9)} = 18.34$, $p < 0.01$; group × treatment: $F_{(1, 9)} = 16.77$, $p < 0.01$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, #$p < 0.05$ and ##$p < 0.01$ vs. saline-treated OBX group (n = 3–5 per group).
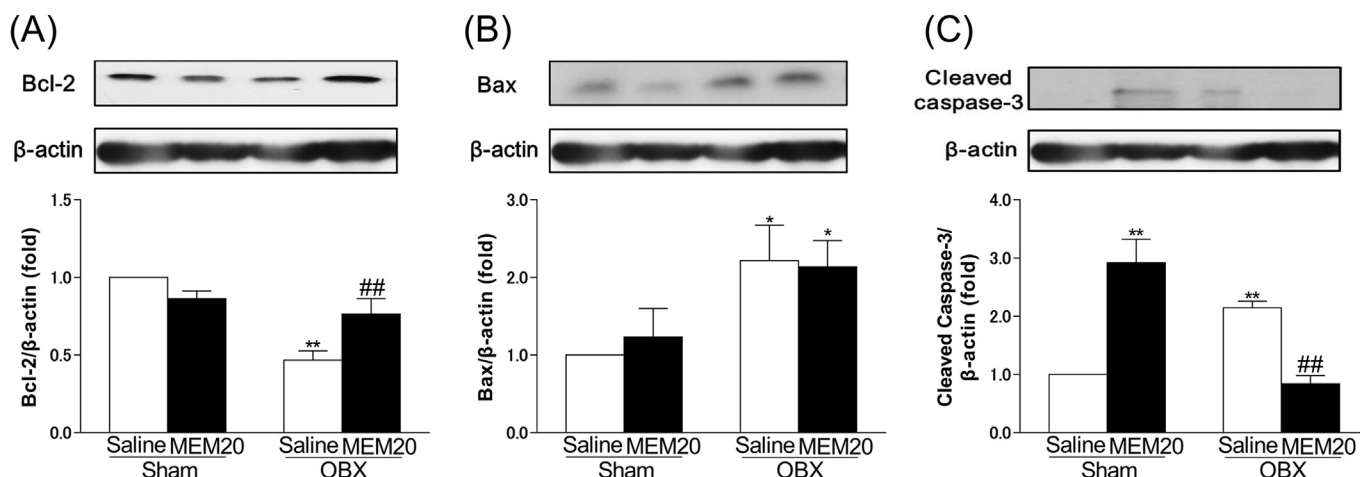
Fig. 8. Altered Bcl-2, Bax, and cleaved caspase-3 levels in the hippocampus after memantine (MEM) administration. A-C: Representative immunoblots probed with antibodies against Bcl-2, Bax, cleaved caspase-3, and β-actin, as indicated. Graphs indicate the quantification of the normalized values of Bcl-2, Bax, and cleaved caspase-3 levels with β-actin. Bars represent means ± SEM. Two-way ANOVA [(A), group: $F_{(1, 15)} = 29.13$, $p < 0.01$; treatment: $F_{(1, 15)} = 1.89$, $p > 0.05$; group × treatment: $F_{(1, 15)} = 13.83$, $p < 0.01$; (B), group: $F_{(1, 15)} = 9.61$, $p < 0.01$; treatment: $F_{(1, 15)} = 0.048$, $p > 0.05$; group × treatment: $F_{(1, 15)} = 0.21$, $p > 0.05$; and (C), group: $F_{(1, 18)} = 3.08$, $p > 0.05$; treatment: $F_{(1, 18)} = 7.06$, $p < 0.05$; group × treatment: $F_{(1, 18)} = 84.44$, $p < 0.01$]. *$p < 0.05$ and **$p < 0.01$ vs. saline-treated sham group, ##$p < 0.01$ vs. saline-treated OBX group (n = 4–6 per group).

Torres-Platas et al., 2014; Setiawan et al., 2015). Thus, the available evidence indicates that dysfunction of microglia is a core event in depression. In the present study, we observed for the first time that OBX mice show significantly increased TNF-α and IL-6 levels, possibly via the activation of p-IκB-α and p-NF-κB p65 and reflected by the increased immunoreactivity for Iba1 (a marker of microglia) and GFAP (a marker of astrocytes) in the hippocampus. Dominguez-Meijide et al. (2017) demonstrated that DA inhibits activated microglia in vitro. Moreover, the neuroprotective effects of MEM observed in primary midbrain neuron-glia culture studies are partly mediated via alternative mechanisms, which reduce microglia-associated inflammation (Wu et al., 2009). We found that microglia-induced inflammation, observed in the hippocampus of OBX mice, was attenuated by MEM, suggesting that MEM exhibits anti-inflammatory effects by inhibiting microglial activity in these mice.

In human neutrophils, activation of NF-κB seems to control spontaneous apoptosis and antiapoptotic effects. The modulation of this pathway most likely regulates the balance between pro- and anti-apoptotic factors, especially members of the Bcl-2 family, (Akgul et al., 2001), thus affecting neutrophil survival. The balance between Bcl-2 (an anti-apoptotic protein) and Bax (a pro-apoptotic protein) regulates apoptotic cell death. Moreover, cleaved caspase-3 is crucial in the process of apoptosis, and its activation contributes to the irreversible stage of apoptosis (Fiandalo and Kyprianou, 2012). Our results showed that MEM reverses the decreased levels of Bcl-2 in the hippocampus of OBX mice, but not the increased levels of Bax. In contrast, MEM reverses the increased levels of cleaved caspase-3 in the hippocampus of OBX mice. Interestingly, sham mice treated with MEM also showed enhanced cleaved caspase-3 levels in the hippocampus. These paradoxical results may be associated with the increased DCX and unchanged NeuN expression levels in the hippocampus of sham mice. Indeed, a previous study reported that the disequilibrium between neuronal proliferation and apoptosis may be involved in the pathogenesis of neuropsychiatric disorders (Genius et al., 2012). We hypothesize that the MEM-induced excess of immature neurons in the hippocampus of sham mice did not differentiate to mature neurons, but instead underwent apoptosis. These results suggest that MEM might partly modulate apoptosis by regulating caspase-3 and the balance between Bcl-2 and Bax in the hippocampus of OBX mice.

Besides the hippocampus, the pathogenesis of depression affects various brain regions, including the prefrontal cortex, striatum, and amygdala (Treadway and Zald, 2011). In this study, we only examined the antidepressant effects of MEM on the hippocampus. Moreover, the regulation of emotional behavior is thought to rely more on ventral hippocampal neurogenesis (Tanti et al., 2012). Hence, further experiments examining the relationship between other brain areas involved in depressive-like behavior, including the ventral hippocampus, and the effects of MEM will be presented in a subsequent paper.

When we investigated the time-dependency of MEM effects in OBX mice by emotional behavioral testing, we observed that the OBX-induced emotional behavior increase was reduced after 4 weeks of MEM administration. A previous study suggested that antidepressants may exert their action by stimulating neurogenesis in the hippocampus (Santarelli et al., 2003). Moreover, enhancement of neurogenesis was observed after chronic but not acute administration, consistently with the time course for the therapeutic action of antidepressants (Malberg et al., 2000). Chiu et al. (2015) reported that the enhancement of neurogenesis and antidepressant effects of L-DOPA (L-3,4-dihydroxyphenylalanine), the precursor of the neurotransmitter DA, or of pramipexole, a D_2/D_3 receptor agonist, were also observed after chronic, but not acute administration. These findings suggest that the dopaminergic activity-related enhancement of neurogenesis, as well as the antidepressant effects, develop later (Chiu et al., 2015). Thus, we suggest that the delayed antidepressant effect of MEM might contribute to the enhancement of neurogenesis via the enhancement of dopaminergic activity. Moreover, recent short-term clinical and meta-analysis studies have reported that MEM as adjunct to sertraline, but not MEM alone, efficiently improves depressive symptoms in patients with major depressive disorder (Amidfar et al., 2017; Kishi et al., 2017). In contrast, a 12-week course of an open-label, flexible-dose study of MEM revealed its efficacy in such patients (Ferguson and Shingleton, 2007). In the present study, MEM exhibited an antidepressant effect 4 weeks after the first treatment in OBX mice. Previous studies have reported that OBX-induced hyperemotional behaviors improve by administration of classic antidepressants, such as desipramine, milnacipran, and fluvoxamine, for 7 days (Saitoh et al., 2003, 2007). Antidepressants are the mainstay of pharmacological treatment for patients with

depressive disorders; however, evidence supporting their use in dementia is unclear (Ford and Almeida, 2017). Thus, we consider that MEM may be effective for depression and emotional disturbances in dementia but its effects might develop later than in the case of classic antidepressants. Therefore, long-term studies on MEM effects on depression are required. However, we suggest that MEM can reduce polypharmacy, a frequent problem in the elderly, which can lead to worsened cognition, increased risk of adverse drug reactions, and falls (Beer et al., 2011).

In conclusion, our results indicate that MEM has antidepressant effects in OBX mice. The mechanism of action may involve the enhancement of cell proliferation in the hippocampus, stimulated by increased BDNF levels and mediated by the activation of the DA receptor signaling pathway (PKA-ERK-CREB). This leads to increased DA via the enhancement of TH activity and inhibition of DA turnover following MEM treatment. In addition, we showed that MEM exerts neuroprotective effects in the hippocampus, by decreasing TNF-α and IL-6 levels and activating the IκB-α and NF-κB p65 signaling pathways. This process is likely mediated by the suppression of microglial activity, and the modulation of the balance between Bcl-2 and Bax, involved in the regulation of apoptotic cell death via caspase-3 activity. Our results provide the basis for the development of MEM strategies not only towards treating dementia, but also depression.

Conflicts of interest

We do not have any conflicts of interest in connection with this manuscript.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.04.013.

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