http://dx.doi.org/10.5607/en.2013.22.1.45
Exp Neurobiol. 2013 Mar;22(1):45-50.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

Experimental Neurobiology

Effects of Serotonin on Erythropoietin Expression in Mouse Hippocampus

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Serotonin (5-hydroxytryptamine, 5-HT), a monoamine neurotransmitter, regulates neurological functions such as mood, sleep, and appetite. Erythropoietin (EPO) is well known for erythropoiesis but has recently emerged as a therapeutic agent in brain diseases. However, the mechanisms that induce EPO in the brain remain unclear. The present study was undertaken to investigate whether the effects of 5-HT involve EPO in murine hippocampal neurons. 5-HT produced a significant increase in neuronal differentiation of hippocampal neural progenitor cells. Expression of erythropoietin was increased in 5-HT-treated cells as well. The actions of 5-HT and EPO appeared to be similar in neurite outgrowth and spine formation. In addition, we show that hippocampal expression of EPO was decreased by chronic unpredictable stress (CUS) and that antidepressant treatment to maintain 5-HT concentration in synaptic cleft reversed this effect. In conclusion, actions of antidepressants might involve EPO induction in the brain.

Key words: erythropoietin, depression, hippocampus

INTRODUCTION

Serotonin (5-HT) is a monoamine neurotransmitter in the central nervous system (CNS) [1]. The serotonergic neurons of the CNS regulate mood, sleep and cognitive functions including memory and learning [2]. 5-HT also plays critical roles in brain development by regulating neurite outgrowth and cell survival [3]. In patients with depression, the level of 5-HT is reduced in the brain, especially hippocampus [4, 5]. It has been suggested that neural atrophy and reduced neurogenesis are key factors in clinical development of depression. The results are supported by the evidence that brain derived neurotrophic factor (BDNF), which plays a pivotal role in neurite outgrowth and neuronal survival, is involved in the antidepressant actions [6]. Numerous studies have revealed that 5-HT increases neurogenesis in the rat brain [7, 8]. Selective serotonin reuptake inhibitors (SSRIs) increase extracellular concentration of 5-HT as inhibitor of the 5-HT reuptake [9]. Among SSRIs, fluoxetine is most widely used to treat depression and stimulates strong neurogenesis in hippocampus [10] where it generates neurons continuously throughout life [11].

EPO was originally described as regulating the synthesis of red blood cells but has recently been suggested as a therapeutic agent in brain disease [12, 13]. A recent study shows that EPO and EPOR are expressed in the central nervous system and that EPO is produced by neurons [14]. EPOR is also expressed in murine hippocampus and cortex [15]. EPO, through binding to its receptor, protects neuronal apoptosis, and increases the expression of BDNF in hippocampus [16]. EPO exerts neuroprotection in neurodegenerative animal model [17]. The neuroprotective effects of EPO are exerted by increasing the level of BDNF and neurogenesis in hippocampus [16]. From the previous studies, it is

Received February 27, 2013, Revised March 6, 2013,
Accepted March 6, 2013

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speculated that there might be a link between actions of EPO and 5-HT in the brain.

We hypothesized that some effect of 5-HT could be mediated by production of EPO. Thus, we investigated whether fluoxetine that maintains concentration of 5-HT in extracellular space as a SSRI, increases EPO in hippocampus, thereby mediating antidepressant efficacy.

MATERIALS AND METHODS

Hippocampal neural progenitor cell culture

Hippocampi were dissected from embryonic day 14 (E14) mouse embryos into HBSS without calcium or magnesium. Cells were plated on 10-cm dishes coated with 15 μg/ml poly-L-ornithine and 1 μg/ml fibronectin (Invitrogen, Carlsbad, CA, USA) at 2.5×10^4 cells/cm^2 in N2 medium and incubated at 37°C in 95% air 5% CO_2. Basic fibroblast growth factor (bFGF, 20 μg/ml, R&D system, Minneapolis, MN, USA) and epidermal growth factor (EGF, 20 μg/ml, R&D system, Minneapolis, MN, USA) were added daily for 2-3 days in order to expand the population of proliferative progenitors, and the medium was changed every 2 days at the time of bFGF and EGF addition. For high-density cultures, the cells on 80% were subcultured once in N2 medium in the presence of 6×10^4 cells/cm^2 in a 24 well plate and these subcultured cells were designated as "passaged once (P1)". The neural progenitors were induced to differentiate by withdrawing growth factors (bFGF, EGF) and kept in a differentiation medium (neurobasal medium add to B27) for 3-5 days. The medium was changed every 3 days. VPA (0.5 μM), 5-HT (10 μM), EPO (10 μg/ml) was treated on 3-5 days following induction of differentiation.

Quantitative RT-PCR

Total RNA from cells was prepared by Trizol reagent (Life technologies Inc., Rockville, MD, USA). For reverse transcription (total reaction volume of 20 μl), 1 μg total RNA was reversely transcribed into cDNA at 70°C for 10 minutes with a mixture of Oligo (dT) 12-18 primer (Bioneer, Daejeon, South Korea) in 10 μl DEPC-treated water. The reaction solution was mixed with Improm-II 5X buffer (Promega, Madison, WI, USA), 10 mM dNTP (Enzynomics, Daejeon, South Korea), RNAsin (Promega, Madison, WI, USA) at 42°C for 60 minutes. For quantitative analysis, real-time PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was performed with iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

The following PCR primers were employed:

EPO sense, 5'-ACT CCG AAC ACT CAC AGT GGA TAC-3',

antisense, 5'-GAT TCT GAG GCT CTT CTT CTC TGG-3'.

Western blot analysis

Protein extracts were mixed with sample loading buffer (62.5 mM Tris-HCL pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) and boiled for denaturation. The samples were then electrophresed on 10% polyacrylamide gels and transferred onto nitrocellulose membrane filters. Blots were blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TTBS) for 1 h, incubated for overnight at 4°C with either rabbit polyclonal EPO (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde/0.15% picric acid in PBS for 20 minutes. The following primary antibody was used. β-tubulin type III (Tuj1) monoclonal at 1:500 (Covance, Emeryville, CA, USA), microtubule-associated protein-2 (MAP2) monoclonal at 1:500 (Sigma-Aldrich, Saint Louis, MO, USA) were used for detecting. For detection of primary antibodies, anti-mouse IgG (Cell Signaling Technology, Beverly, MA, USA) were used according to the specification of the manufacturer. Cells were mounted in the vectashield mounting medium for fluorescence and photographed with a fluorescent microscope (Nikon, Tokyo, Japan).

Spine density and dendritic length analysis

Images were acquired through Z-stacks, which typically consists of 10 scans at high zoom at 1-μm steps in the z axis. Each MAP2 (+) neuron was definitely distinguishable from other cells. For an analysis of Spine density, we focused on first-order dendrite from cells. For each cell, 3 dendritic segments were used for a spine analysis. Spine counting was conducted with 60X objective using the Delta Vision Images (Applied Precision, Seattle, USA). The number of spines was counted in a 10 μm segment. For an analysis of dendritic arborization, the Z-trace feature was used to measure the three-dimensional length of the dendritic arbor. With each cell, first-order dendrites arising from the cell bodies were separately traced. They were measured by Image J.

Animals and drug treatment

Two-month-old male C57BL/6 mice were housed in a 12-h light/dark cycle animal facility. Mice were daily injected intraperitoneally with saline or fluoxetine (5 mg/kg) for 14 days.

Intracerebroventricular (i.c.v) microinjection

Mice were implanted with a guide cannula into the lateral ventricles (coordinates±0.9 mm (anterior/posterior), ±0.34 mm (lateral), and ±2.15 mm (ventral) relative to the bregma). After a 7 day recovery period, 5-HT (1 μM, 1 μl) was delivered at the rate of

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0.25 μl/min with an injection cannula protruding 0.5 mm beyond the guide cannula. The injection cannula stayed in the guide cannula for 10 min after infusion.

Chronic unpredictable stress (CUS)

Chronic unpredictable stress (CUS) is an experimental procedure in which animals are exposed to a variable sequence of mild and unpredictable stressors. The chronic unpredictable stress was performed similar to published protocol [18]. These sequences of stressors were reported reduction of peripheral brain-derived neurotrophic factor (BDNF) (Table 1) [18]. We used the C57BL/6 mice. Mice were group housed (3-4 mice/cage) and were applied to different stressors per day for 14 days.

Table 1. Chronic unpredictable stress

Type of stressor and duration	Occurrence during 14 days
Light on overnight	1, 7, 9, 12 (4)
Cold 4°C 1 hr	2, 5, 6, 11 (4)
Cage tilt 45° overnight	6, 11, 13 (3)
Light off 3 hr	2, 9, 10, 12 (4)
Food deprivation	2, 8, 14 (3)
Cage rotation 1 hr	3, 5, 7, 12 (4)
Stroboscope overnight	3, 8, 10, 13 (4)
Restraint 1 hr	4, 6, 7, 9 (4)
Odor overnight	7, 11 (2)
Swim stress 18°C	5, 10, 13, 14 (4)
Cage tilt 45° 3 hr	2, 3, 4, 8 (4)

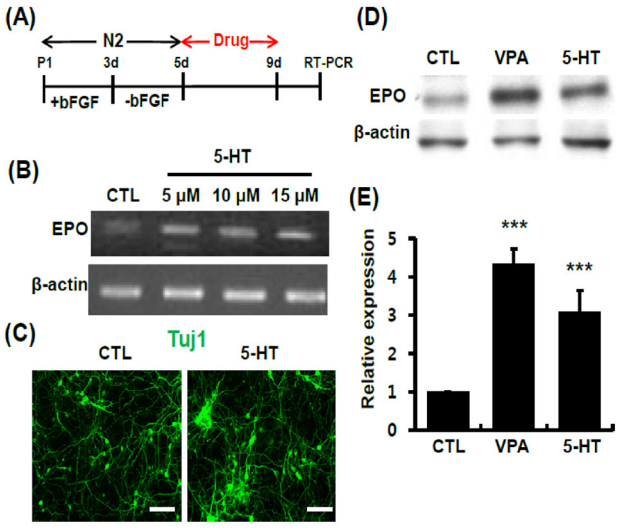

Fig. 1. EPO expression is increased by 5-HT in neuronal differentiation of hippocampal neural progenitor cells. (A) Hippocampal neural progenitor cells are grown in NB27 media containing growth factors (bFGF, EGF). Cells were treated with 10 μM, 50 μM or 100 μM of 5-HT for 4 days or left untreated in the absence of growth factors. (B) Total RNA was analyzed for expression of EPO by RT-PCR. (C) Treatment of 5-HT increased the expression of Tuj1, a neuron marker. (D) Cell lysates were prepared and analyzed by western blotting for the expression of EPO. Hippocampal neural progenitor cells were treated with 5-HT (10 μM), VPA (0.5 μM) for 4 days. (E) EPO expression was increased by VPA and 5-HT treatment. The EPO expression was normalized to the amount of β-actin and is represented as fold inductions relative to the untreated cells. The values represent the mean±SEM (n=3, VPA, 4.34±0.38; 5-HT, 3.07±0.57, ***p<0.001). Scale bars, 30 μm.

Statistical analysis

All data were measured by Image J software and were expressed as mean±SEM. Statistical significance was determined by Student's *t*-test.

RESULTS

To examine the effect of 5-HT on neuronal differentiation, hippocampal neuronal progenitor cells were incubated with

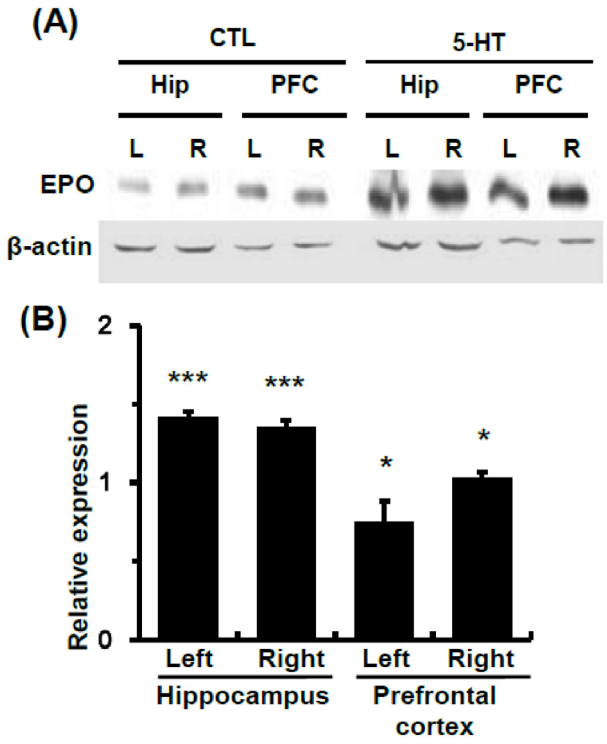

Fig. 2. EPO is induced by 5-HT in the hippocampus of mice. Cannula was implanted into the lateral ventricles. The concentration of 5-HT was 1 μM (1 μl). The mice were sacrificed 24 h after the injection. (A) The expression of EPO at the levels of protein was evaluated through Western blotting. (B) The expression of EPO was more pronounced in the hippocampus than in the prefrontal cortex (n=3, Hippocampus Left, 1.42±0.03; Hippocampus Right, 1.34±0.04; Prefrontal cortex, Left 0.75±0.13; Prefrontal cortex Right, 1.03±0.03, *p<0.05, ***p<0.001). The expression of EPO was normalized to the amount of β-actin and is represented as fold inductions relative to the saline-treated brains.

5-HT in the absence of bFGF for 4 days (Fig. 1A). EPO mRNA expression was increased by 5-HT ranging 5 to 15 μM by RT-PCR (Fig. 1B). Therefore, we used 5-HT at 10 μM concentration *in vitro* (Fig. 1C). Given the induction of EPO by 5-HT in the absence of bFGF, we asked whether 5-HT treatment during differentiation would increase neuronal differentiation. To test this, cells were subject to 5-HT treatment during differentiation and their neuronal differentiation and were compared by immunocytochemistry using an antibody specific for a neuronal marker. 5-HT significantly increased the percentage of neurons expressing β-tubulin III (Tuj1) in progenitor cell cultures (Fig. 1C). These results suggest that 5-HT treatment is sufficient to induce neuronal differentiation as well as EPO induction. We detected an increase in the EPO in the protein extracts. The increase was comparable to that of valproic acid (VPA) which promotes neuronal differentiaiton as previously indicated [19, 20]. Treatment with either VPA (0.5 mM) or 5-HT (10 μM) for 4 days increased the protein level of EPO (Fig. 1D, E: VPA, 4.34±0.38; 5-HT, 3.07±0.57, ***p<0.001).

To determine whether EPO is induced upon 5-HT *in vivo*, we injected 5-HT into the mice brain through intracerebroventricular (i.c.v.) injection. Mice were sacrificed 24 hr after the injection and the expression of EPO was evaluated (Fig. 2A). EPO mRNA was increased in the hippocampus and prefrontal cortex of 5-HT treated animals (Fig. 2B: hippocampus left, 1.42±0.03; hippocampus right, 1.35±0.05; prefrontal cortex left, 0.75±0.13; prefrontal cortex right, 1.03±0.03, *p<0.05, ***p<0.001). These results indicate that EPO was induced by 5-HT *in vivo* as well.

We previously reported that EPO and VPA enhance neurite outgrowth, respectively [19]. To examine whether 5-HT has similar effects, neurite outgrowth was quantified by measuring the lengths of branches extending from MAP2(+) cell soma. The dendritic lengths of MAP2(+) neurons were significantly increased by 5-HT treatment (Fig. 3A). Quantitative analysis revealed that treatment with 5-HT increased the lengths of dendrites comparable to those in the EPO- or VPA-treated cells (Fig. 3B: in μm, CTL, 147.19±19.03; VPA 200.94±24.31, 5-HT 206.65±7.62, EPO 241.25±13.92, *p<0.05, ***p<0.001). To test if 5-HT can also promote the spine formation, we examined spine number in cells treated with 5-HT (Fig. 3C). Treatment cells with 5-HT significantly increased the number of spine in MAP2(+) cells, similarily to the effects of EPO or VPA (Fig. 3D: CTL, 2.28±0.21; VPA, 3.65±0.43; 5-HT, 3.54±0.15; EPO, 3.85±0.39, ***p<0.01) (Fig. 4D). Taken together, these results suggest that 5-HT and EPO enhance the neurite outgrowth and the spine formation during the neuronal differentiation.

To investigate if the systemic effects of fluoxetine involve EPO, we used a chronic unpredictable stress (CUS) model, a putative

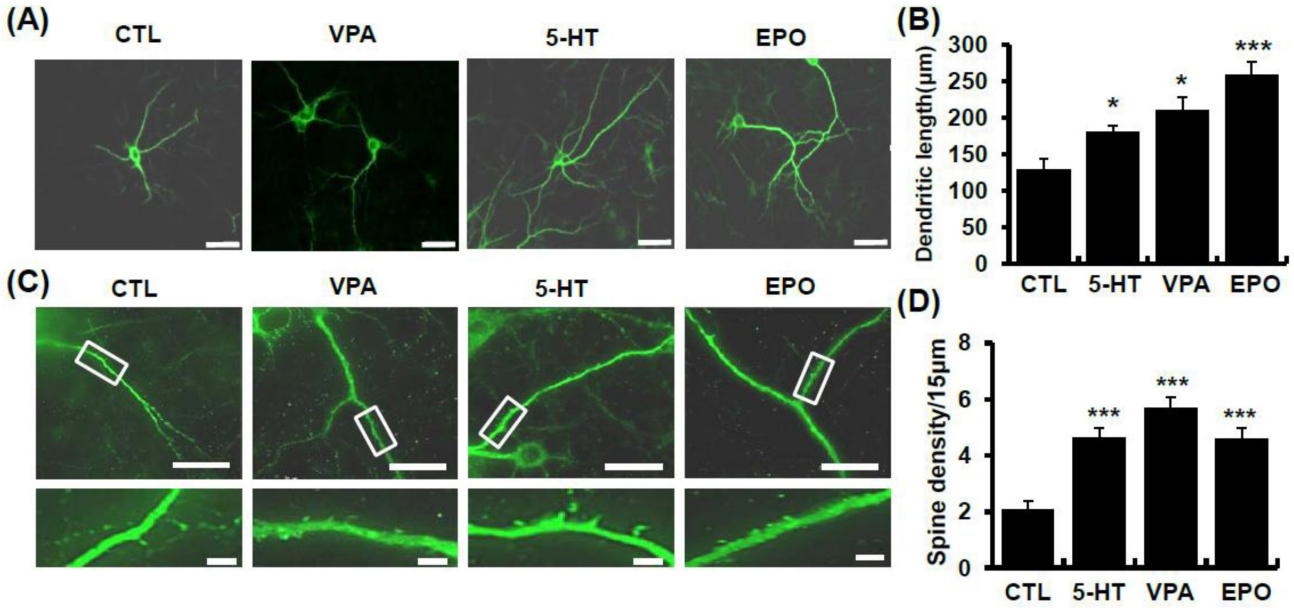

Fig. 3. The length of dendrite and the density of spine are enhanced by EPO. Hippocampal neural progenitor cells were treated with 5-HT (10 μM), VPA (0.5 mM) or EPO (10 μg/ml) in the absence of growth factors for 4 days. (A) Representative images of MAP2(+) cells. (B) Immunocytochemistry analysis shows that the length of dendrites was increased by VPA, 5-HT or EPO (n= 15~25 cells, CTL, 147.19±19.02; VPA, 200.94±24.31; 5-HT, 206.66±7.62; EPO, 241.25±13.92, *p<0.05, ***p<0.001). (C) Representative images of the magnified Z-stack projections of the spines of MAP2(+) cells. (D) The density of the spines was significantly increased by VPA, 5-HT or EPO (n=10~15 cells, CTL, 2.28±0.21; VPA, 3.65±0.43; 5-HT, 3.54±0.15; EPO, 3.85±0.39, ***p<0.001). Scale bars, 30 μm (A), 5 μm (C).

http://dx.doi.org/10.5607/en.2013.22.1.45

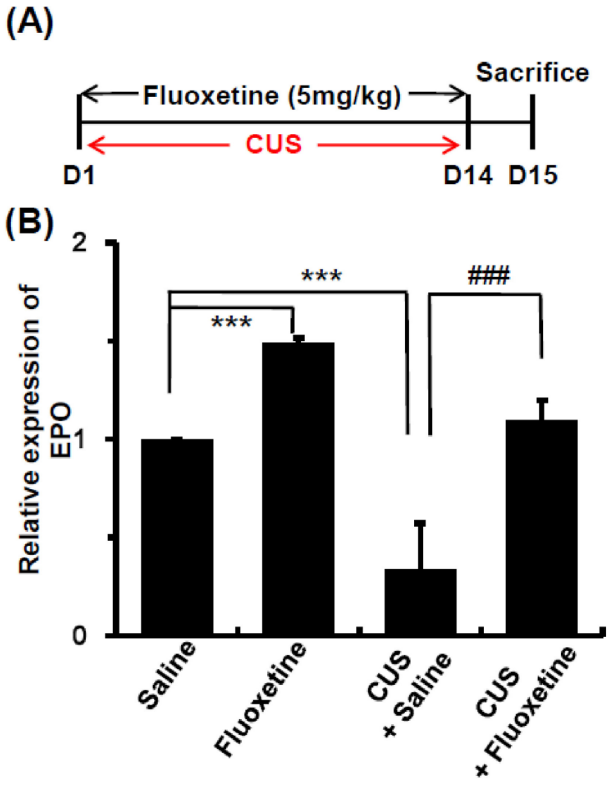

Fig. 4. EPO downregulation is recovered by fluoxetine in CUS animals. (A) Mice were exposed to chronic unpredictable stresses for 14 days and injected with fluoxetine (5 mg/kg) once a day at the same time. (B) Hippocampal tissues from the mice were analyzed for EPO expressions by quantitative RT-PCR. The expression of EPO mRNA was increased in fluoxetine group and was decreased in CUS group. The decrease in EPO mRNA in CUS animals was recovered by fluoxetine. The expression of EPO was normalized to the amount of β-actin and is represented as fold inductions relative to the untreated cells (fluoxetine, 1.49±0.03; CUS, 0.34±0.32; CUS + fluoxetine, 1.10±0.14, $^{***}p<0.001$, $^{###}p<0.001$).

animal model of depression. If antidepressant action of fluoxetine involves EPO, the level of EPO expression might be decreased and fluoxetine treatment should recover the EPO expression in CUS animals. Chronic fluoxetine treatment alone had a significant effect on neuritin expression in non-stressed animals (Fig. 4B: $^{***}p<0.001$). EPO mRNA levels are significantly decreased in the hippocampus of CUS animals (Fig. 4B: $^{***}p<0.001$). In contrast, chronic administration (2 wk, initiated at day 1 of CUS) of the 5-HT selective reuptake inhibitor, fluoxetine, significantly reversed the effects of CUS exposure in the hippocampus (Fig. 4B: fluoxetine, 1.49±0.02; CUS, 0.34±0.23; CUS+FLX, 1.10±0.09, $^{***}p<0.001$, $^{###}p<0.001$).

DISCUSSION

Our present study demonstrates that EPO is induced by 5-HT and fluoxetine recovers the downregulation of EPO in the hippocampus of CUS animals. Our results suggest that EPO might act as a downstream molecule in 5-HT signaling pathways in the hippocampus. EPO induction upon 5-HT pulse might contribute to the EPO-mediated cell proliferation and neurogenesis in hippocampus, as previously reported [19]. However, the molecular mechanisms underlying 5-HT induction of EPO need further investigations.

Prefrontal cortex and hippocampus are involved in working memory [21]. In patients with major depression, lower volume of hippocampus and prefrontal cortex has been found [22]. Injection of 5-HT into the lateral ventricles produces EPO induction in the hippocampus and prefrontal cortex with a greater extent in the hippocampus, suggesting that hippocampus might be a primary target area of EPO in the brian.

Previous results showed that neuronal outgrowth is decreased by the long term depression (LTP) of hippocampus [23] and stress [24]. In the present study, we showed that EPO increases the dendrite length and the spine density, consistent with previous results showing EPO stimulates the neurite outgrowth in polarizing hippocampal neurons [25].

Chronic unpredictable stress (CUS) is an well-documented animal model for depression [26]. *in vivo* data shows that fluoxetine increases the EPO expression in the hippocampus of CUS animals. The EPO expression was decreased in CUS animals, which was restored by fluoxetine. These findings are in line with our *in vitro* data in that the treatment of neuronal differentiation of hippocampal neural progenitor cells with 5-HT increased the EPO levels. In addition, these results indicate that antidepressant effects of fluoxetine are possibly mediated by signalings involving EPO. In conclusion, these results indicate that 5-HT can increase the EPO expression in mice hippocampus and EPO expression is restored by fluoxetine in CUS mouse model.

ACKNOWLEDGEMENTS

This research was supported by the National Research Foundation of Korea (NRF) grant (No. 2011-0028317) funded by the Ministry of Education, Science and Technology (MEST), Republic of Korea (HS).

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