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
Miyeon Choi and Hyeon Son 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 2.5×10⁴ cells/cm² in N2 medium and incubated at 37°C in 95% air 5% CO₂. 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 6×10⁴ cells/cm² 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 (Enzymonics, 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:

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 electrophoresed 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
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.

**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

| 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.

**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 differentiation 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, similarly 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<0.001) (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, 206.65±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).
EPO Induction by 5-HT

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.

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 brain.

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 a 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).

REFERENCES

1. Green AR (2006) Neuropharmacology of 5-hydroxytryptamine. Br J Pharmacol 147 Suppl 1:S145-S152.
2. Zamani A, Qu Z (2012) Serotonin activates angiogenic phosphorylation signaling in human endothelial cells. FEBS
Lett 586:2360-2365.

3. Gaspar P, Cases O, Maroteaux L (2003) The developmental role of serotonin: news from mouse molecular genetics. Nat Rev Neurosci 4:1002-1012.

4. Coppen A (1967) The biochemistry of affective disorders. Br J Psychiatry 113:1237-1264.

5. Duman RS, Nakagawa S, Malberg J (2001) Regulation of adult neurogenesis by antidepressant treatment. Neuropsychopharmacology 25:836-844.

6. Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weissstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

7. Breznun JM, Daszuta A (1999) Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. Neuroscience 89:999-1002.

8. Gould E (1999) Serotonin and hippocampal neurogenesis. Neuropsychopharmacology 24:465-51S.

9. Riad M, Zimmel L, Rbahi L, Watkins KC, Hamon M, Descarries L (2004) Acute treatment with the antidepressant fluoxetine internalizes 5-HT1A autoreceptors and reduces the in vivo binding of the PET radioligand [18F]MPPF in the nucleus raphe dorsalis of rat. J Neurosci 24:5420-5426.

10. Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

11. Leunen B, Gould E, Shors TJ (2006) Is there a link between adult neurogenesis and learning? Hippocampus 16:216-224.

12. Mohamed HE, El-Swefy SE, Mohamed RH, Ghanim AM (2013) Effect of erythropoietin therapy on the progression of cisplatin induced renal injury in rats. Exp Toxicol Pathol 65:197-203.

13. Noguchi CT, Asavaritikrai P, Teng R, Jia Y (2007) Role of erythropoietin in the brain. Crit Rev Oncol Hematol 64:159-171.

14. Brines M, Cerami A (2005) Emerging biological roles for erythropoietin in the nervous system. Nat Rev Neurosci 6:484-494.

15. Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R (1997) Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 76:105-116.

16. Viviani B, Bartesaghi S, Corsini E, Villa P, Ghezzi P, Garau A, Galli CL, Marinovich M (2005) Erythropoietin protects primary hippocampal neurons increasing the expression of brain-derived neurotrophic factor. J Neurochem 93:412-421.

17. Brines ML, Ghezzi P, Keenan S, Agnelo D, de Lanerolle NC, Cerami C, Itri LM, Cerami A (2000) Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 97:10526-10531.

18. Schmidt BD, Duman RS (2010) Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models. Neuropsychopharmacology 35:2378-2391.

19. Oh DH, Lee IY, Choi M, Kim SH, Son H (2012) Comparison of neurite outgrowth induced by erythropoietin (EPO) and carbamylated erythropoietin (CEPO) in hippocampal neural progenitor cells. Korean J Physiol Pharmacol 16:281-285.

20. Yu IT, Park JY, Kim SH, Lee JS, Kim YS, Son H (2009) Valproic acid promotes neuronal differentiation by induction of proneural factors in association with H4 acetylation. Neuropsychopharmacology 35:473-480.

21. Izaki Y, Takita M, Akema T (2008) Specific role of the posterior dorsal hippocampus-prefrontal cortex in short-term working memory. Eur J Neurosci 27:3029-3034.

22. Frodl T, Meisenzahl E, Zetzsche T, Bottlender R, Born C, Groll C, Läger M, Leinsinger G, Hahn K, Möller HI (2002) Enlargement of the amygdala in patients with a first episode of major depression. Biol Psychiatry 51:708-714.

23. Sdrulla AD, Linden DJ (2007) Double dissociation between long-term depression and dendritic spine morphology in cerebellar Purkinje cells. Nat Neurosci 10:546-548.

24. Fricker AD, Rios C, Devi LA, Gomes I (2005) Serotonin receptor activation leads to neurite outgrowth and neuronal survival. Brain Res Mol Brain Res 138:228-235.

25. Ransome MI, Turnley AM (2008) Erythropoietin promotes axonal growth in a model of neuronal polarization. Mol Cell Neurosci 38:537-547.

26. Katz RJ, Roth KA, Carroll BJ (1981) Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. Neurosci Biobehav Rev 5:247-251.