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Carbamylated erythropoietin promotes neurite outgrowth and neuronal spine formation in association with CBP/p300

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ABSTRACT

Both erythropoietin (EPO) and carbamylated EPO (cEPO) have been shown to increase the length of neurites and spine density in neurons. However, the molecular mechanism underlying the EPO- and cEPO-induced neuronal differentiation has yet to be investigated. To address this issue, we investigated epigenetic modifications that regulate gene expression in neurons. Neurons treated with EPO or cEPO display an upregulation of E1A-binding protein (p300) and p300-mediated p53 acetylation, possibly increasing the transactivation activity of p53 on growth-associated protein 43 (GAP43). Treatment of cells with cEPO markedly increases spine formation and potentiates p300-mediated transactivation of PSD95, Shank2 and 3 compared to EPO. These results demonstrate that cEPO controls neuronal differentiation via acetylation of transcription factors and subsequent transactivation of target genes. These findings have important medical implications because cEPO is of interest in the development of therapeutic agents against neuropsychiatric disorders.

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1. Introduction

Erythropoietin (EPO) and the EPO receptor (EpoR) have also been demonstrated in the central nervous system of animals and humans [1], and they are essential for neurodevelopment, adult neurogenesis and neuroprotection [1,2]. High expression of EPO and EpoR in the hippocampal formation suggests that EPO plays an important role in hippocampal functioning [3,4]. Carbamylated EPO (cEPO) is an EPO derivative without hematopoietic bioactivity and that does not bind to homodimeric EpoR [5]. Previous work has demonstrated that EPO and cEPO promote axonal and dendritic growth in hippocampal neuronal cultures [6–8]. cEPO exerts its functions through binding to the β common receptor but not to the classical EpoR [5], but the specific cellular mechanisms remain largely unknown. Recently, it was shown that acetylation of transcription factor (TF) p53 at specific lysines in its C-terminus promotes neurite and axonal regeneration [9,10]. CREB-binding protein (CBP)/E1A-associated protein (p300) is a histone acetyltransferases (HAT) that acetylates histones and in turn modulates neuronal functions [10], and has been associated with the regulation of neuronal fate [11,12].

To obtain insights into the molecular mechanisms of neurite outgrowth and spine formation mediated by EPO and cEPO, we focused on epigenetic modification by the lysine acetyltransferases CBP/p300 of factors that are directly involved in neuronal differentiation. Then, we specifically examined whether CBP/p300 could upregulate TFs that promote axonal outgrowth and spine density.

2. Materials and methods

2.1. Rat hippocampal neuronal cell culture and drug treatment

Sprague–Dawley adult pregnant female rats (Harlan Sprague Dawley, Indianapolis, IN) and the conditions used by Son et al. [13] were used with modifications. Briefly, hippocampi were dissected from embryonic day 16.5 (E16.5) embryos into Hank's balanced salt solution (HBSS) without calcium or magnesium. The dissociated cells were plated on 10-cm-diameter 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 ng/ml, R&D Systems Inc, Minneapolis, MN, USA) was

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added daily for 2–3 days, and the medium was changed every 2 days at the time of bFGF addition. For high-density cultures, cells at 80% confluence were subcultured and induced to differentiate in a differentiation medium (Neurobasal medium: NB) for 3–5 days. Cells were incubated in the presence of EPO (0, 5, 10, 20 ng/ml) (R&D Systems Inc.), or cEPO (0, 5, 10, 20 ng/ml) (H. Lundbeck A/S, Denmark) for 3 days.

2.2. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde/0.15% picric acid in PBS for 20 min. The following primary antibodies were used: for neuronal differentiation, monoclonal anti-β-tubulin type III (Tuj1) at 1:200 (Covance, Berkeley, CA, USA) and polyclonal anti-microtubule associated protein-2 (MAP2) at 1:400 (Abcam, Cambridge, UK). For detection of primary antibodies, cells were incubated in PBS containing Alexa Fluor 488-conjugated anti-goat IgG (Molecular Probes, Eugene, OR, USA) secondary antibodies for 1 h at room temperature. They were then mounted in Vectashield mounting medium and photographed under a confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Western blot analysis

Equal amounts of protein extracts were mixed with SDS sample buffer and boiled for denaturation. The samples were then electrophoresed on 10% polyacrylamide gel and transferred onto nitrocellulose membrane filters (Amersham Pharmacia Biotech, UK). Blots were blocked with 5% non-fat milk in TTBS (0.05% Tween-20 in 1X TBS) for 1 h and incubated overnight at 4 °C with primary antibodies. The primary antibodies used are indicated in Supplementary informations.

2.4. Measurement of spine density and neurite outgrowth

Images of MAP2(+) cells were acquired through Z-stacks, which typically consisted of 10 scans at high zoom at 1-μm steps in the *z* axis using the Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany). For analysis of spine density, we focused on first-order dendrites from cells. For each cell, 3 dendritic segments were used for spine analysis. The final value was averaged from 9 to 15 cells per group and expressed as the number of spines/10 μm. For analysis of neurite outgrowth, the Z-trace feature of Tuj1(+) cells was used to measure the three-dimensional length from 40 to 50 cells per group. The length of neurite outgrowth was defined as the distance from the soma to the tip of the longest branch. For statistical analyses, at least 10 random fields were counted for each condition.

2.5. Coimmunoprecipitation and chromatin immunoprecipitation (ChIP)

Cells were lysed in RIPA buffer, and scraped. Extract was diluted to 500 μl in RIPA buffer and incubated with 1–2 μg of antibody for 1 h to overnight at 4 °C. 20 μl of protein A/G-agarose was added to the IP reaction (Roche Applied Sciences, Mannheim, Germany), and the mix was incubated for 2 h at 4 °C. The beads were washed three times in RIPA buffer: 1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, resuspended in SDS–polyacrylamide gel loading buffer, and proteins were analyzed by Western blotting. ChIP assays were performed using the EZ-ChIP kit (Millipore, Billerica, MA, USA) in accordance with the manufacturer's instructions. Purified DNA samples were normalized and subjected to PCR analysis. The PCR primers employed are indicated in Supplementary informations.

2.6. Statistical analysis

All experiments were performed at least three times. Optical densities were measured using Image J software. Quantified data are presented as mean ± SEM. The significance of differences was assessed by an unpaired Student's *t*-test.

3. Results

3.1. Effects of EPO and its carbamylated derivative on phosphorylation of STAT3, ERK and Akt

We previously reported that both EPO and cEPO treatment significantly increase the length of neurites and spine density in MAP2(+) cells [8]. However, the molecular mechanisms underlying the effects of EPO and cEPO remain unclear. To assess this issue, we first examined whether EPO and cEPO differentially regulate the activation of STAT3, ERK and Akt, which are signaling molecules involved in neurite outgrowth [14,15]. In initial experiments, we ascertained that neuronal differentiation was efficiently induced in the presence of EPO or cEPO with a peak at a concentration of 10 ng/ml when cells were stimulated for up to 72 h. Western blots of cultured hippocampal neural progenitor cells treated with either EPO or cEPO at a concentration of 10 ng/ml showed that phosphorylation of STAT3 was indeed increased, with a peak in phosphorylation at 24 h post-treatment (Fig. 1A and B; EPO, $^{**}p < 0.01$; cEPO, $^{***}p < 0.001$). An increase in p-ERK levels was highly significant 72 h after EPO or cEPO treatment (Fig. 1B; EPO, cEPO, $^{*}p < 0.05$). Treatment of cells with EPO or cEPO caused an increase in p-Akt with a peak at 48 h of application (Fig. 1B; EPO, cEPO 48 h, $^{*}p < 0.05$). In addition, the increase in the level of β-tubulin type III (Tuj1), a neuronal marker, was similar after 72 h in cells treated with EPO and cEPO (Fig. 1B; EPO, $^{*}p < 0.05$; cEPO, $^{**}p < 0.01$), suggesting that EPO and cEPO stimulate a similar acquisition of the neuronal phenotype. These results indicate that both EPO and cEPO at a concentration of 10 ng/ml resulted in prominent activation of STAT, ERK, and AKT with similar pharmacokinetics. Hippocampal neural progenitor cells were treated with EPO or cEPO at a concentration of 10 ng/ml for 72 h for all subsequent experiments.

Given these findings, we decided to further examine whether increased activation of signaling molecules was associated with neuronal differentiation including neuronal outgrowth in hippocampal neural progenitor cells. We previously demonstrated that EPO and cEPO increase neurite outgrowth and spine density [8]. In the present study, we reexamined the effects of EPO and cEPO on neuronal differentiation since we used cEPO provided by Lundbeck instead of EPO that was carbamylated in our laboratory as previously described [16]. Neurite outgrowth was quantified by measuring the lengths of branches extending from MAP2(+) cell somas. MAP2(+) cells exhibited complex branching patterns compared to those in the control group when neural progenitor cells were incubated with EPO or cEPO (Fig. 2A). Quantitative analysis revealed that treatment with EPO or cEPO significantly increased the lengths of dendrites compared to those in the vehicle-treated controls (Fig. 2B; EPO, cEPO, $^{*}p < 0.05$). We determined the number of dendritic branch points, a frequently-used morphometric parameter, in MAP2(+) neurons. The number of dendritic branch points was increased by treatment with EPO and cEPO (Fig. 2C; EPO, cEPO, $^{**}p < 0.01$). The axonal lengths of Tuj1(+) neurons were also increased by EPO and cEPO treatment (Fig. 2F; EPO, cEPO, $^{**}p < 0.01$). These results indicate that EPO and cEPO cause a similar enhancement in neurite outgrowth. To test if EPO and cEPO can also promote spine formation, we examined spine number. Neurons formed in the presence of cEPO gave rise to dendrites with

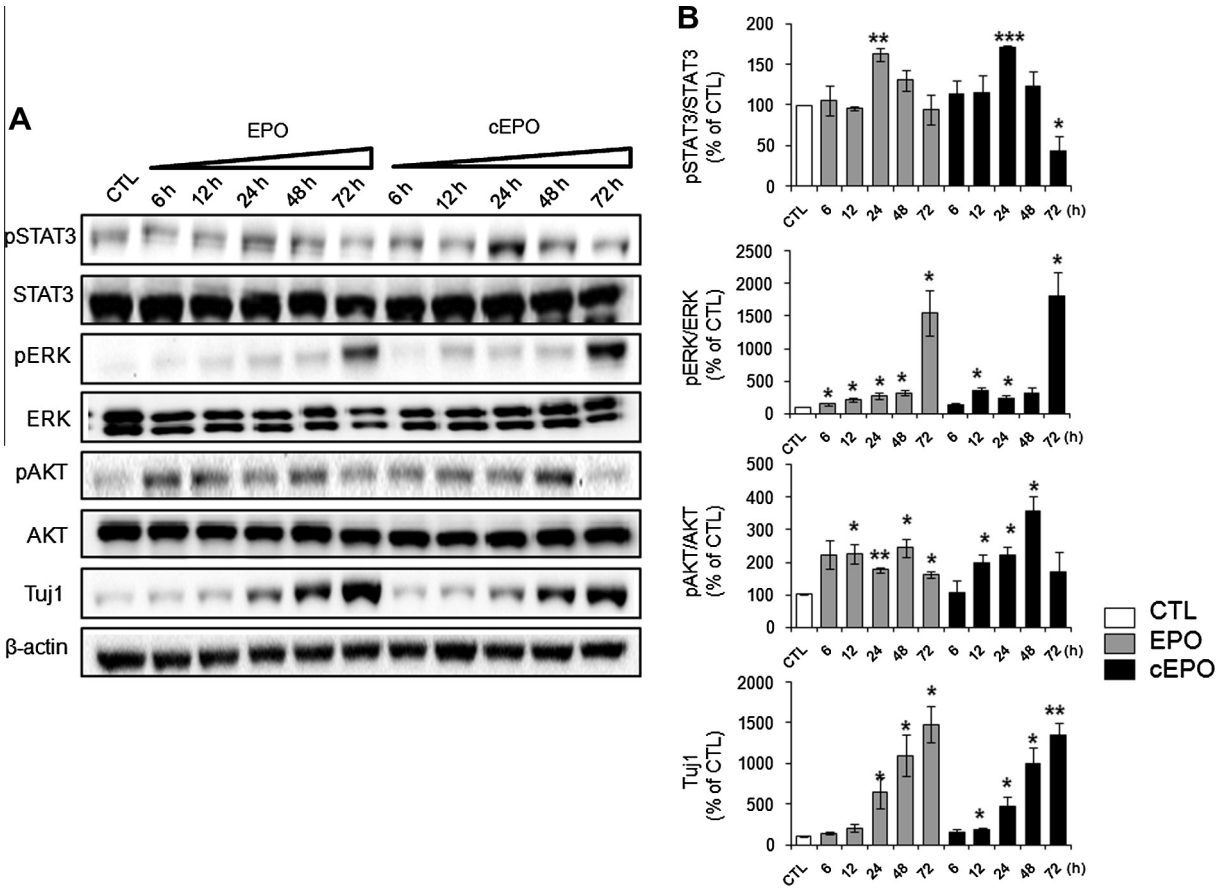

Fig. 1. EPO- and cEPO-dependent activation of signaling molecules by Western blotting analysis. (A) Representative blots. EPO (10 ng/ml) activates PI3K, ERK Akt and STAT3 in a time-dependent manner, with peaks at approximately 24–72 h after treatment. (B) Quantitative analyses of activation shown in A. Optical density (OD) of each molecule was normalized to the OD of total protein or β-actin. Expression levels at each time point (or treatment) are depicted relative to the level of the vehicle-treated control (CTL) for comparison. $n = 4$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

a greater spine density than those in control or EPO-treated cells (Fig. 2D).

To verify whether the effects observed with EPO and cEPO were specific to its function in the expression of synaptic molecules, we determined levels of synapsin I and PSD95 by Western blotting. Treatment with EPO or cEPO at a concentration of 10 ng/ml resulted in a similar enhancement in synapsin I and PSD95 after 72 h of treatment (Fig. 2G and H: $^{**}p < 0.01$). We examined two other well-characterized post-synaptic molecules, Shank2 and 3. Immunoblotting analysis showed that both EPO and cEPO trigger the expression of Shank2 and 3 (Fig. 2G and H: $^{*}p < 0.05$). Neurons treated with the EPO and cEPO also showed an enhancement in neurite outgrowth. Therefore, we asked whether treatment with EPO or cEPO would lead to an increase in GAP43, a well-described stimulator of axonal outgrowth. Our experiments revealed highly significant increases in GAP43 levels 72 h after EPO or cEPO treatment (Fig. 2G and H: $^{*}p < 0.05$).

Taken together, these results indicate that cEPO promotes neuronal signaling and neurite outgrowth during the process of neuronal differentiation in hippocampal neural progenitor cells under basal conditions at least as efficiently as does EPO.

3.2. EPO and cEPO upregulate CBP/p300

To obtain insights into the molecular mechanisms of neurite outgrowth and spine formation mediated by EPO and cEPO, we investigated whether the expression of TF and cofactor genes are controlled by EPO and cEPO. It has been demonstrated that acetylation of the TF p53 is required for neurite outgrowth and that acetylated-p53 (Ac-p53) induces transcription of GAP43 [17]. We found that p53 protein levels remained similar to control in the presence of EPO or cEPO. However, p53 acetylation was highly and significantly upregulated in cells treated with EPO or cEPO 72 h after treatment (Fig. 3A and B, respectively: $^{*}p < 0.05$, $^{**}p < 0.01$). It has been demonstrated that p53 is actetylated by CBP/p300 and P/CAF [18], which are also essential transcriptional co-factors involved in overall neuronal function [19]. Given that Ac-p53 was increased by EPO and cEPO, we further investigated whether CBP/p300 and P/CAF were increased in cells treated with EPO or cEPO. Expression of p300 and P/CAF proteins was highly and significantly upregulated in cells treated with EPO and cEPO (Fig. 3A and B, respectively: $^{*}p < 0.05$). Interestingly, when we measured global changes in H3 K9-14 acetylation in neurons, we found a significant increase (Fig. 3A and B: $^{***}p < 0.001$). H3 K9-14 acetylation is known to be mediated by the HATs, CBP/p300 and P/CAF [10], so this result is consistent with the upregulation of p300 and P/CAF seen in the presence of EPO or cEPO. Therefore, these results suggest that EPO and cEPO might affect neuronal differentiation by enhancing transcription of specific promoters via CBP/p300 and that EPO and cEPO might activate p53-dependent pro-outgrowth signaling pathways.

Because CBP/p300 is associated with P/CAF and p53, we proceeded to examine a potential interplay between p300 and P/CAF or p53 in the presence of EPO or cEPO. Comparisons of binding data sets showed that a fraction of p300 bound by P/CAF was increased in the presence of cEPO. However, treatment with EPO resulted in a

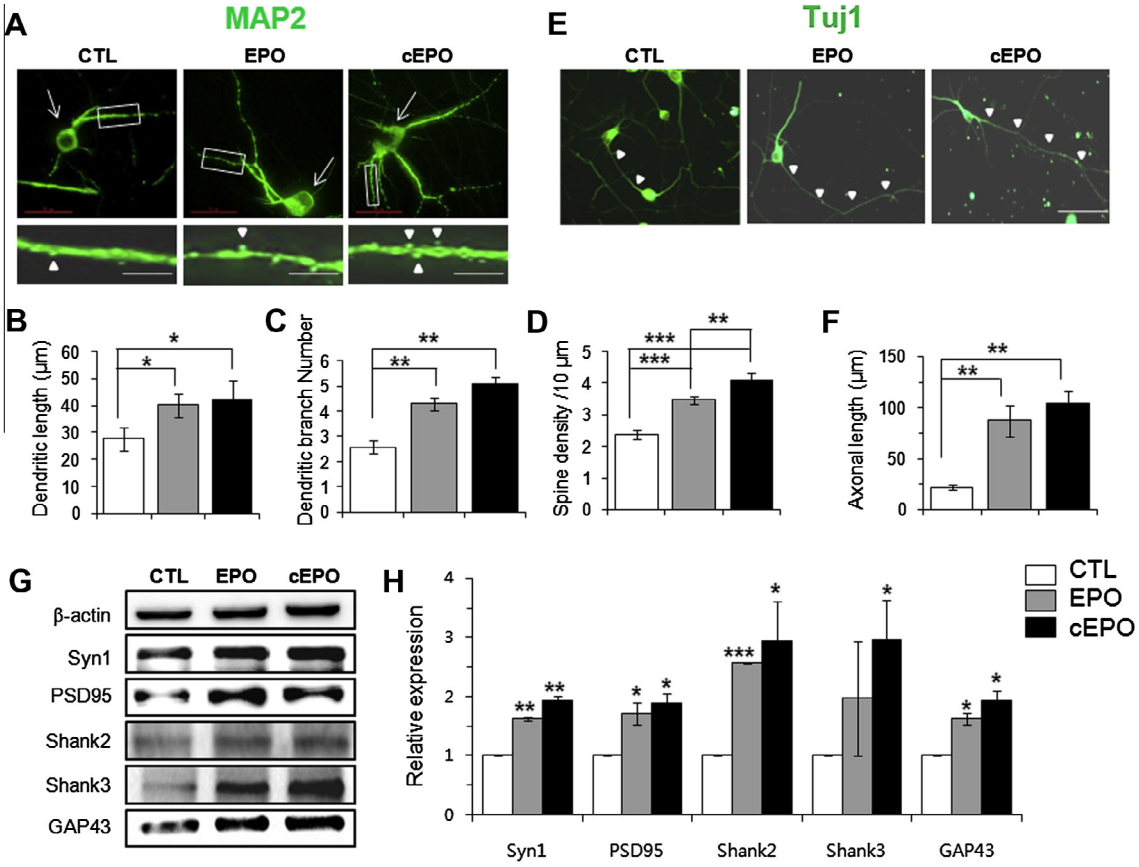

Fig. 2. EPO and cEPO increase the lengths of neurites and the spine density. The lengths of dendrites and axons extending from the cell soma (arrows) were counted in neurons immunostained for MAP2 (B) and Tuj1 (E), respectively (n = 40–50 cells per treatment). (A) Representative images of dendritic projections of MAP2(+) hippocampal neurons. (Low) Representative images are shown of high-magnification Z-stack projections of the spine density designated in the box. (B, C) Treatment with EPO and EPO significantly increased dendritic outgrowth ($^{*}p < 0.05$) and number of dendritic branches ($^{**}p < 0.01$) compared with those of the CTL. (D) The density of dendritic spines (arrowheads) was significantly increased by EPO and cEPO ($^{***}p < 0.001$ compared to CTL, $^{**}p < 0.01$, comparison between EPO and cEPO). (E) Representative images of high-magnification Z-stack projections of axons (arrowheads) in Tuj1(+) neurons. (F) Length of axons was increased by EPO and cEPO ($^{**}p < 0.01$). (G) Representative blots. (H) OD of each molecule was normalized to the OD of β-actin and is represented as ratio relative to CTL (n = 4 per group from 4 discrete cultures). Scale bar: 30 μm (A, top panel), 5 μm (A, low panel), and 30 μm (E). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

less marked increase in the association of p300 and P/CAF than did treatment with cEPO (Fig. 3C and D). Furthermore, coimmunoprecipitation assays revealed that the association of the p300 and Ac-p53 in nuclear extracts was significantly increased in the presence of cEPO. EPO showed only a tendency of increase in the association between p300 and Ac-p53. (Fig. 3C and D).

To directly demonstrate binding of p300 to the promoters of GAP43, ChIP assays were carried out in hippocampal neural progenitor cells treated with either EPO or cEPO. Results showed that enrichment of p300 at GAP43 promoter was more efficiently increased by cEPO than by EPO (Fig. 3E and F). We also found that treatment with cEPO induced more enrichment of p300 at CBP promoter than did EPO treatment (Fig. 3E and G). Together, these experiments show that after EPO or cEPO treatment, p53 is hyperacetylated and the expression of the specific p53-dependent pro-outgrowth target GAP43 is induced by increased CBP/p300/Ac-p53 promoter occupancy. cEPO was more efficient than EPO in all of these processes.

3.3. cEPO upregulates p300-dependent transactivation of PSD95, Shank2 and Shank3

Having obtained evidence that EPO and cEPO induce upregulation of PSD95, Shank2 and Shank3, we further investigated whether CBP/p300 could affect the transcriptional activation of these genes implicated in synaptic formation/plasticity. ChIP assays demonstrated that cEPO prominently and significantly increased the occupancy of p300 on the promoters of PSD95, Shank2, and Shank3, and EPO had a similar but less pronounced effect (Fig. 4).

These results suggest that cEPO might promote synaptic development more efficiently than EPO, as evidenced by an increased spine density seen upon morphological observation of hippocampal neural progenitor cell differentiation.

4. Discussion

The aim of this study was to compare the ability of EPO and cEPO to promote neuronal differentiation and to assess factors which are related to neurite outgrowth and spine formation in cultured hippocampal neurons. Our work shows for the first time that EPO and cEPO increase H3 K9-14 as well as p53 acetylation. EPO and cEPO also induce association of p300 with CBP and GAP43 promoters. cEPO promotes not only neurite outgrowth, but also stimulates spine formation by induction of gene expression via localization of p300 on the promoters of PSD95, Shank2, and Shank3. Importantly, cEPO was more effective than EPO at inducing the association of p300 with the promoters of genes implicated in synaptic formation/plasticity. This finding is consistent with the observed increase in spine density in these cells.

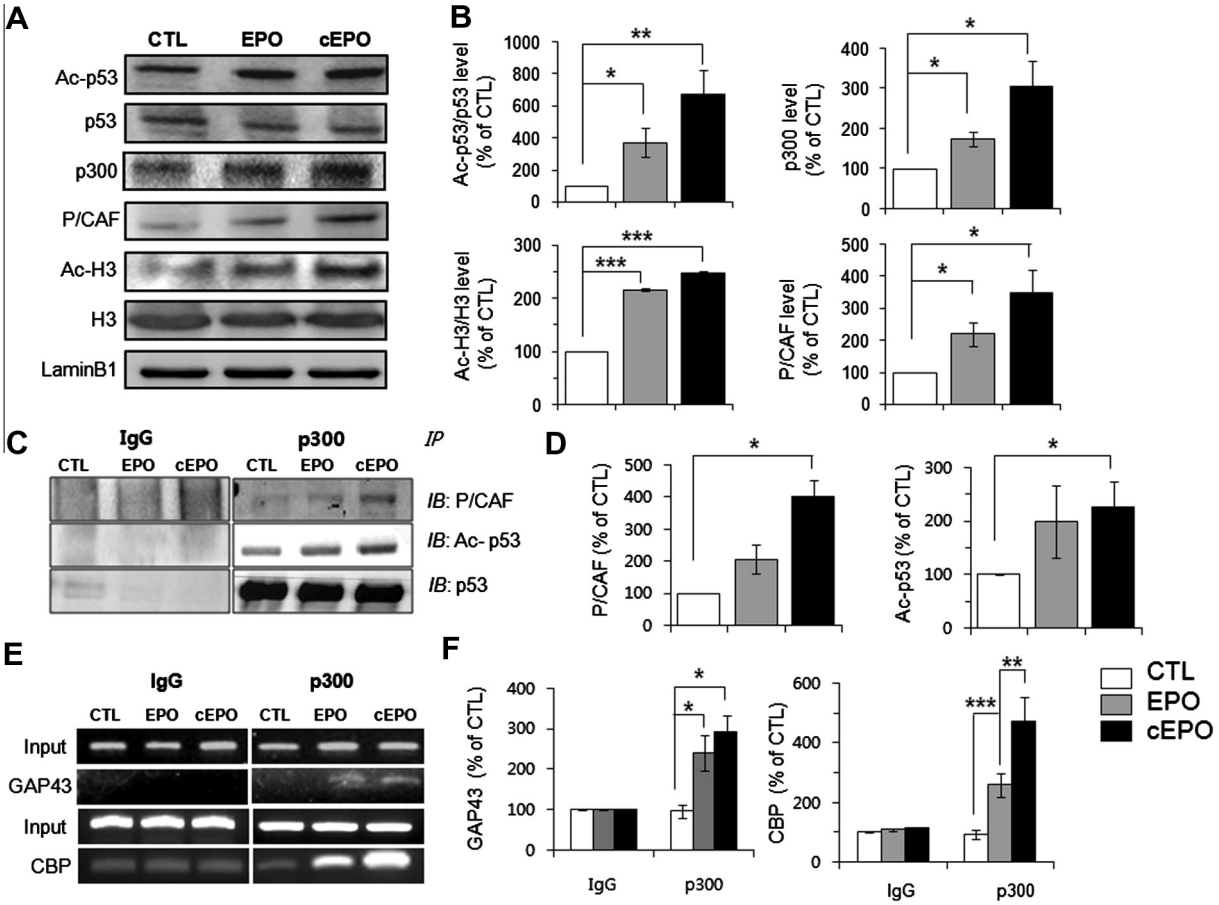

Fig. 3. EPO and cEPO increase p300, Ac-p53, and Ac-H3 levels and promote transactivation of GAP43. (A) Representative immunoblots of p300, P/CAF, Ac-p53, and Ac-H3. (B) The upregulation of p300, P/CAF and Ac-p53 observed after 10 ng/ml EPO or cEPO treatment versus vehicle is represented in the bar graph of densitometry. OD of each molecule was normalized to the OD of laminB1 and is represented as % change relative to CTL ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 4$ per group from 4 discrete cultures). (C) Coimmunoprecipitation assay. The interaction between p300 and P/CAF, Ac-p53 or p53 was analyzed. The abundance of Ac-p53, p53 and P/CAF was determined by immunoblotting (*IB*) using antibodies, respectively. (D) The physical interaction between p300 and P/CAF or Ac-p53 was increased when cEPO was added ($^{*}p < 0.05$, $n = 3$ per group from 3 discrete cultures). (E) ChIP assay. (F) The occupancy of p300 on the promoters of GAP43 and CBP was highly increased at 72 h in culture treated with EPO or cEPO compared to vehicle ($^{*}p < 0.05$, $^{***}p < 0.001$; $^{**}p < 0.01$, comparison of EPO and cEPO). The promoter occupancy upon each treatment was normalized to the amount of input and is represented as % change relative to cells that were vehicle-treated and immunoprecipitated with IgG. $n = 3$ independent experiments run in triplicate.

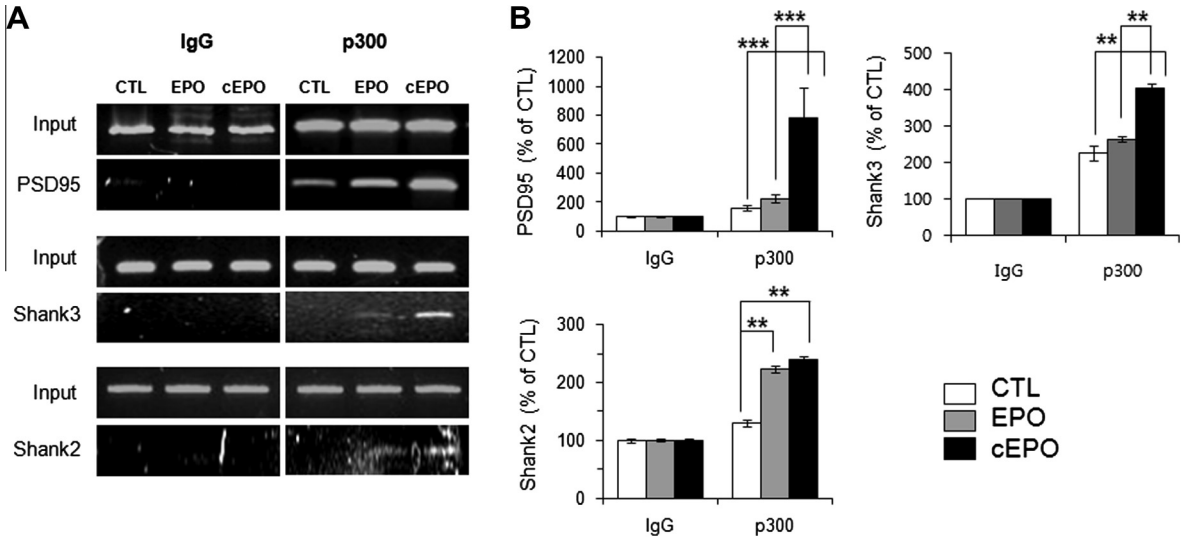

Fig. 4. cEPO promotes transactivation of PSD proteins. (A) ChIP assay. Immunoprecipitation was performed on cells treated with EPO or cEPO with antibodies for p300, and levels of the PSD95, Shank2, and Shank3 promoters in the immunoprecipitates were measured by RT-PCR. (B) PSD95, Shank2 and Shank3 promoters are more prominently occupied by p300 in cells treated with cEPO than in cells receiving EPO or vehicle treatment. The promoter occupancy upon each treatment was normalized to the amount of input and is represented as % change relative to cells that were vehicle-treated and immunoprecipitated with IgG. $n = 3$ independent experiments run in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$.

Unlike those of EPO, the cellular mechanisms responsible for the neuritogenic effects of cEPO have not been fully elucidated. Our results suggest that a general chromatin remodeling of the GAP43 gene promoter by p300 is involved both in EPO and cEPO-induced neuritogenesis because p300 expression and p300 transactivation of GAP43 promoter were increased either by EPO or cEPO treatment. p300 is likely to act via p53 acetylation, and the importance of p300 acetylation is supported by the results of IP assays in which Ac-p53 is highly associated with p300 in the presence of cEPO, whereas albeit modestly associated in the presence of EPO. It has been suggested that p53 may act as a gatekeeper between cellular quiescence and differentiation in both neurogenesis and neurite outgrowth [20]. EPO and cEPO might trigger the p53 action during neuronal differentiation. Given our previous findings that EPO and cEPO increase hippocampal neurogenesis [8], further studies are warranted to investigate whether the chromatin remodeling mediated by EPO and cEPO may play a crucial role in their actions in neuronal differentiation during embryonic development and adulthood. Our results do not establish whether other neuritogenic TFs, such as plasticity-related genes (PRGs/LPPRs) and Krüppel-like factor (KLFs) [21,22], are influenced by EPO- and cEPO-induced histone acetylation and other chromatin remodeling (Fig. 3A).

No studies have yet implicated CBP, p300 and the closely-related P/CAF in the neuronal outgrowth-stimulating effects of EPO and cEPO. Here, we have shown that p300 and P/CAF are upregulated by EPO and cEPO. Specifically, EPO and cEPO promote not only p300 upregulation, but also stimulate CBP gene expression by p300 transactivation of the CBP promoter. This suggests the presence of a positive feedback loop initiated by the relative increase in acetyltransferase activity upon treatment with EPO or cEPO. This leads to histone acetylation and activation of the CBP, p300 and P/CAF promoters, increasing gene expression and further enhancing the histone acetylation.

Because of their neurogenic and neurotrophic activities, EPO and cEPO show tremendous potential as therapeutic agents to treat neuropsychiatric disorders such as depression and anxiety [23,24]. Increases in neuronal differentiation and neurogenesis are ultimately required to treat depression. Despite recent success with drugs that increase neurogenesis, there is a clear need for clinically safe and effective drugs that will increase neuronal differentiation substantially.

In the current study, we found that pharmacological EPO and cEPO enhance CBP/p300 and H3 acetylation, which in turn induces gene expression. Notably, cEPO in particular has a remarkable effect on the spine formation. Although further studies are required to clarify the mechanisms underlying the chromatin modifications in the presence of EPO and cEPO, our results provide a new theoretical basis for developing therapeutic agents affecting neuronal differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.066.

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