Chronic glucocorticoid exposure suppressed the differentiation and survival of embryonic neural stem/progenitor cells: possible involvement of ERK and PI3K/Akt signaling in the neuronal differentiation

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Abstract

Growing evidence suggests that excess glucocorticoids (GCs) exposure during the pregnancy results in behavioral abnormality in offspring. Although research using animal models has demonstrated that systemic GCs treatment impairs development of fetal brain, direct impact of GCs on the phenotype of **embryonic neural stem/progenitor cells (eNSPCs)** and its mechanism has not been fully understood. Here, we investigated the effect of chronic GCs exposure on cell proliferation, differentiation, and survival of **eNSPCs *in vitro***. Corticosterone (CORT, a murine GC) treatment did not affect the proliferation of **eNSPCs**. On the other hand, decreased expression of neuronal, synaptic, and astroglial marker proteins were observed when the differentiation of **eNSPCs** was induced in the presence of CORT. CORT also reduced the survival rate of **eNSPCs** after the differentiation. Moreover, CORT inhibited extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling **pathways**, which were activated during cell differentiation of **eNSPCs**. **Inhibiting these signaling pathways reduced neural differentiation and eNSPCs viability, indicating their essential roles in the eNSPCs differentiation. Furthermore, IGF-I, a potent PI3K/Akt and ERK signaling stimulator, partially restored the adverse effect of CORT on eNSPCs, suggesting a possible involvement of the repression of these intracellular signaling in the GCs-caused eNSPCs impairment.**

Keyword

Glucocorticoid, corticosterone, neural stem cell, differentiation, cell survival, extracellular signal-regulated kinase, phosphatidylinositol 3-kinase

Introduction

Growing clinical evidence shows that maternal stress during pregnancy is associated with the abnormality of behavioral and emotional development in children and the increased risk of psychiatric diseases including attention deficient hyperactivity disorder, depression, schizophrenia, and autism (Graignic-Philippe et al., 2014). In animal studies, it has been demonstrated that prenatal stress causes reduced exploration activity, decreased spatial memory, inability to extinguish conditioned fear memory, increased anxiety, and depression-like behavior (Alonso et al., 1991; Bingham et al., 2013; Lordi et al., 2000; Salomon et al., 2011; Schneider et al., 2002). Although the underlying mechanism of adverse influence of prenatal stress is not completely understood, it has been proposed that excess glucocorticoids (GCs) exposure is involved in the negative effect of prenatal stress on the central nervous system (CNS) function (Salomon et al., 2011; Wilcoxon and Redei, 2007). GCs are secreted from the adrenal gland in response to the stressful conditions, and trigger coping response via activating glucocorticoid receptor (GR) (De Kloet et al., 1998). Normally, fetal GCs levels is retained lower than that of maternal plasma by placental 11 beta-hydroxysteroid dehydrogenase type 2 (11 β -HSD2) which converts active GC into inactive cortisone (Reynolds, 2013). However, the increased maternal serum GCs by maternal stress can overcome the capacity of 11 β -HSD2, and affect the fetus (Reynolds, 2013). Salomon *et al.* (2011) reported that anxiety-like behavior of prenatally-stressed rats was diminished after maternal adrenalectomy with replacement of basal corticosterone (CORT, a murine GC) (Salomon et al., 2011). They also found that CORT injection to adrenalectomised dams reinstated the anxiety-like behavior in their offspring (Salomon et al., 2011). Further, Wilcoxon et al. (2007) showed that female offspring from adrenalectomised rats replaced with high levels of CORT exhibited depression-like behavior similar to the consequences of prenatal stress (Wilcoxon and Redei, 2007).

Importantly, it has been suggested that GCs exposure affects neurogenesis and cell survival in the developing brain. For example, repetitive administration of dexamethasone (DEX, a synthetic GC) to postnatal (P) 4-6 day-old rats caused decreased brain weight and defected neurogenesis in the subgranular zone in hippocampus, the subventricular zone, and cortex at P8 (Kanagawa et al.,

2006). Furthermore, prenatal exposure to DEX impaired brain development of fetus such as reduced volume of hippocampal CA region, decreased number of CA neurons, and increased apoptotic cells in CA and DG areas (Noorlander et al., 2014). Although these animal experiments suggest that systemic GCs exposure influences the development of **embryonic neural stem/progenitor cells (eNSPCs)** which play a major role in CNS development, it is still unclear whether GCs directly impact on **eNSPCs** not through systemic reactions secondary to GCs exposure.

In this study, to approach the direct action of GCs on **eNSPCs** at the cellular and molecular levels, we focused on negative impact of GCs exposure on proliferation, differentiation, and survival of **eNSPCs** using dissociated cultures. In our system, it was revealed that **eNSPCs** exposed to CORT for 3 days during the proliferation phase and subsequent 7 days differentiation phase showed decreased neuronal differentiation and increased cell vulnerability.

Materials and methods

Primary culture of eNSPC

eNSPCs were prepared from embryonic 14 day-old Wistar rats. The telencephalon tissue was dissected and digested with papain solution (phosphate-buffered saline (PBS) containing 9 units/ml papain and 200 units/ml DNase I) for 20 min at 37°C. After dissociation by pipetting, cell debris was removed with a cell strainer (BD Falcon, CA, USA). Then, the cells were diluted with proliferation medium (KBM neural stem cell medium from KOHJIN BIO, Saitama, Japan) containing KBM neural stem cell supplement (which includes EGF and bFGF) at $0.5\text{--}1.5 \times 10^6$ cells/ml, and incubated in T75 flasks (CORNING, NY, USA) for 4 days. Proliferated **eNSPC** generated floating cluster, neurosphere. After 4 days maintenance, neurospheres were collected and digested with trypsin/EDTA solution (Sigma-Aldrich, MO, USA) for 5 min at 37°C. Then, dissociated cells in proliferation medium were plated at a cell density of $5 \times 10^4/\text{cm}^2$ on polyethylenimine-coated 3.5 cm dishes (for western blotting, BD Falcon), 48-well plates (for MTT assay, BD Falcon), or glass-bottom dishes (for immunostaining, Matsunami Glass Ind. Ltd, Osaka, Japan). The cells were maintained for 3 days to observe the effect of drug on cell proliferation. After the proliferation phase, the medium was replaced with KBM neural stem cell medium containing 1/50 volume of B-27 supplement (Life Technologies) as differentiation medium and the cells were cultured for the subsequent 7 days to examine the influence of drug on cell differentiation. At 3 days after the initiating differentiation, the medium was replaced with fresh one. Using this protocol, most of the **eNSPCs** were differentiated to TUJ1-positive neural cells while a few of them were differentiated to glial cells, which corresponds with differentiation identities of **eNSPCs** at early phase of *in vivo* CNS development (Okada et al., 2008). All animal experiments were carried out in accordance with the guidelines of the Animal Ethics Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan.

Drug treatment

CORT (0.001–10 μM , Sigma-Aldrich) was applied to **eNSPC** cultures at 3–5 hours after cell seeding, and the CORT exposure was continued throughout the **eNSPCs**

proliferation/differentiation stage. DEX (Sigma-Aldrich) treatment was performed same as CORT treatment. In all experiments except those shown in Fig.1, CORT and DEX were applied at 0.1-10 μ M because serum CORT concentrations in rats exposed to the stressful condition shows the order of μ M (Cook and Wellman, 2003; Williams et al. 1999). U0126 (10 μ M, Millipore, MA, USA) and LY294002 (10 μ M, Cell Signaling, MA, USA) were applied every other day during the first 1-5 days after the induction of differentiation to inhibit ERK or PI3K/Akt signaling, respectively. Rat recombinant insulin-like growth factor-I (IGF-I, 100 ng/ml, R&D Systems Inc., MN, USA) was applied at 1 day after the initiation of differentiation.

Immunostaining

The effect of CORT or DEX on the cell proliferation was assessed by counting the number of nestin-positive cells. At the end of the proliferation phase, cells were fixed with 4% paraformaldehyde. The fixed cells were incubated with PBS containing 10% FBS, 0.5% Triton-X, and a specific antibody for nestin (Millipore) or SOX2 (Abcam, Cambridge, UK) at 4°C overnight. After washing with PBS, cells were incubated with Alex Fluor 488 mouse IgG_{2A} (Life Technologies) and Alex Fluor 546 rabbit IgG (Life Technologies) at 24°C for 1 hour. Subsequently, the cells were washed with PBS and incubated with 5 mg/ml Hoechst 33342 at 24 °C for 15 min. After washing with PBS, immunoreactivity was observed with Axio Observer.Z1 fluorescence microscopy (ZEISS, Germany).

MTT assay

MTT assay was performed to estimate cell viability as previously reported (Odaka et al., 2014). At the end of proliferation or differentiation, cultured cells were incubated with 250 μ l KBM neural stem cell medium containing MTT (0.5 mg/ml) for 1.5–2.5 hours at 37 °C. Then, the 250 μ l SDS lysis buffer was applied to each well and disrupted by sonication. The absorbance of each sample at 570 nm was measured using an iMark Micro plate reader (Bio-Rad Laboratories Inc., CA, USA). As MTT (yellow compound) is hydrolyzed to formazan (violet) by mitochondrial enzyme in surviving cells, the number of surviving cells can be estimated.

Western Blotting

To examine expression of marker proteins and phosphorylation of signaling molecules, western blotting was performed as previously described (Odaka et al., 2014). Protein concentrations of whole cell lysate were determined using BCA Protein Assay Kit (Thermo Scientific, MA, USA). The same amount of protein from each sample was separated by electrophoresis using 8 % SDS polyacrylamide gel. Separated proteins were electrophoretically transferred to a polyvinylidene fluoride membrane using Semi-Dry Transfer Cell (BioRad). The membrane was blocked by 5% non-fat dried milk, and then incubated with 1% non-fat dried milk containing primary antibody at 4°C overnight. After washing with Tris-Buffered Saline, the membrane was incubated with secondary antibody at room temperature for 1 hour. After washing, targeted protein bands were visualized with ImmunoStar Regents (Wako), and detected by X-ray film (GE Healthcare, WI, USA) or EZ Capture II/ST (ATTO, Tokyo, Japan). Each band density was analyzed using Lane & Spot Analyzer software (ATTO). The following primary antibodies were used: anti-SOX2 (Abcam), anti-TUJ1 (Berkeley Antibody Company, CA, USA), anti-doublecortin (DCX, Cell signaling), anti-syntaxin (Sigma-Aldrich), anti-synapsin I (Millipore), anti-GluR1 (Millipore), anti-GAD65/67 (Sigma-Aldrich), anti-GFAP (Millipore), anti- β -Actin (Sigma-Aldrich), anti-phospho ERK (Thr202/Tyr204) (Cell Signaling), anti-ERK (Cell Signaling), anti-phospho Akt (S473) (Cell signaling), and anti-Akt (Cell signaling) antibodies. The following secondary antibodies were used: Rabbit IgG (H&L) Secondary Antibody Peroxidase Conjugated Properties (Rockland, PA, USA), Peroxidase-AffiniPure Goat Anti-Mouse IgG (Jackson Immunoresearch Labs Inc., PA, USA).

Statistical analysis

All data are expressed as mean $\pm$ SD. Difference between means of two groups was analyzed by Student's *t*-test. Differences between means of multiple groups were analyzed by one or two-way ANOVA (SPSS Japan, Tokyo, Japan) followed by Bonferroni *post-hoc* test. The significance level was set at $P < 0.05$ for all analyses.

Results

CORT exposure did not affect proliferation of eNSPCs

To evaluate possible influence of CORT on the proliferation, eNSPCs were maintained with proliferation medium for 3 days with or without CORT exposure (0.1-10 μ M). As shown in Fig.1A, cell viability determined with MTT assay was not altered by CORT exposure (0.001-10 μ M). Immunostaining with nestin and SOX2 antibody indicated most of the cells expressed these NSPC marker proteins and CORT did not affect the number of nestin-positive eNSPCs after 3 days proliferation. (SupFig. 1A). Long-term treatment with CORT for 7days also did not affect the cell viability (SupFig. 1B). Similarly, DEX also did not change the cell viability (Fig. 1B).

Time-dependent change of marker proteins for differentiation of eNSPCs

Removal of mitogens (EGF and bFGF) from culture medium induces spontaneous differentiation of NSPCs (Kelly et al., 2005). To assess differentiation status of eNSPCs, expression of various cell-type specific marker proteins for NSPCs (SOX2), neurons (TUI1 and DCX), synapses (syntaxin, synapsin I, and GluR1), GABAergic neurons (GAD65/67), and astroglial cells (GFAP) was determined. While SOX2 expression was gradually decreased, TUI1, DCX, syntaxin, and GFAP were increased from 3 days after the initiation of differentiation (Fig. 1C, SupFig. 2A). Furthermore, marked increase of synapsin I and GAD65/67 levels were observed at 5 days and increased expression of GluR1 was detected at 7 days (Fig. 1C, SupFig. 2A).

CORT impaired differentiation and survival of eNSPCs

To evaluate the effect of CORT on the differentiation of eNSPCs, we determined the expression levels of these marker proteins at 3 and 7 days after the induction of differentiation. eNSPCs exposed to 10 μ M CORT showed lower expression of TUI1, DCX, syntaxin, and synapsin I (but not SOX2) at 3 days compared with control (Fig. 2A, SupFig. 2B). Furthermore, GFAP expression was reduced by CORT exposure (0.1-10 μ M) in a dose-dependent manner (Fig. 2A).

At 7 days, levels of neural and synaptic proteins (except TUJ1) were decreased by 10 μ M CORT (Fig. 2B, SupFig. 2C). GFAP was also reduced by 1-10 μ M CORT application (Fig. 2B).

Next, we assessed the effect of CORT on cell viability during differentiation. As shown in Fig. 2C, 10 μ M CORT caused a slight but significant reduction in cell viability at 7 days (not 3 days) after the induction of differentiation. Notably, decreased neuronal, synaptic, and astroglial proteins were also observed in DEX-treated **eNSPCs** (Fig. 3A, B). Moreover, exposure of 0.1-10 μ M DEX decreased cell viability at 7 days (Fig. 3C).

*CORT suppressed activation of ERK and PI3K/Akt signaling during **eNSPCs** differentiation*

To clarify the underlying mechanism of the CORT-caused impairment in **eNSPCs** differentiation, we focused on ERK and PI3K/Akt signaling pathways because activation of these signaling pathways is supposed to be involved in differentiation and/or survival of neural cells (Ishii et al., 2010; Qiao et al., 2012). We found that levels of activated (phosphorylated) ERK1/2 (pERK1/2) and pAkt were increased with the progression of differentiation (Fig. 4A). Remarkably, 10 μ M CORT decreased ERK2 phosphorylation levels at 3 days (Fig. 4B). The level of activated Akt was also decreased by the exposure of 1-10 μ M CORT (Fig. 4B). At 7 days after the start of differentiation, 10 μ M CORT reduced both pERK2 and pAkt levels (Fig. 4C). Interestingly, required dose of CORT for inhibiting ERK and PI3K/Akt signaling at 3 and 7 days is consistent with those in disruption of neural differentiation and survival (see Fig. 2).

*Increased activity of ERK and PI3K/Akt signaling was essential for differentiation and cell survival of **eNSPC***

To investigate the possible role of ERK and PI3K/Akt signaling activation in the differentiation and survival of **eNSPC**, chronic treatment with specific inhibitor for ERK or PI3K/Akt signaling pathways (U0126 and LY294002, respectively) was performed. Although the application of U0126 resulted in a drastic decrease of pERK1/2 at 3 and 7 days after the differentiation initiation, only GAD65/67 expression at 7 days was reduced (Fig. 5A, B). On the other hand, LY294002 reduced Akt phosphorylation levels, which in turn suppressed expression of DCX, syntaxin, and

GFAP at 3 days (Fig. 5C). LY294002 also decreased expression of DCX, syntaxin, and GAD65/67, but not GFAP at 7 days (Fig. 5D). These results suggest that PI3K/Akt signaling would regulate initiation of astrogliogenesis and contribute to the progress of neuronal differentiation, while ERK signaling activation is involved in the differentiation for GABAergic neurons. Furthermore, a significant decrease in the cell viability of eNSPC at 3 days was caused by LY294002. At 7 days, both U0126 and LY294002 reduced the cell viability (Fig. 5E).

IGF-I treatment restored the CORT-induced impairment of eNSPC differentiation

To further examine the involvement of PI3K/Akt and ERK signaling suppression in the CORT-induced impairment of eNSPCs differentiation, IGF-I, a potent stimulator of PI3K/Akt and ERK signaling pathways, was applied to NSPCs with or without CORT treatment. IGF-I partially restored the suppression of pAkt, but not pERK1/2, in CORT-treated eNSPC (Fig. 6A). In addition, IGF-I reversed the CORT-caused downregulation of DCX and syntaxin without affecting GFAP expression, suggesting that PI3K/Akt signaling at least partially contributes to neural differentiation and synaptogenesis (Fig. 6B).

Discussion

In the present study, we examined the direct impact of GCs exposure on the phenotype of eNSPCs using *in vitro* system. We found that CORT exposure decreased levels of neuronal, synaptic, and astroglial specific proteins at 3 and 7 days after the initiation of differentiation. Furthermore, CORT treatment reduced the cell viability at 7 days (but not 3 days). It was also demonstrated that CORT treatment suppressed ERK and Akt signaling during differentiation stage. Especially, LY294002, a PI3K/Akt signaling inhibitor, also suppressed expression of neuronal and synaptic marker proteins, implying that the downregulation of Akt signaling in the presence of GCs could be accountable for the reduced neural differentiation.

CORT exerts its biological effect by transcriptional regulation via activating mineralocorticoid receptor (MR) and/or GR. Because CORT binds to MR with about 10 times higher affinity than to GR, MR is predominantly occupied under basal blood level of CORT (De Kloet et al., 1998). The

elevated level of CORT under stressful condition leads to the activation of GR, contributing to physical stress response (De Kloet et al., 1998). Because relatively high dose (10 μ M) of CORT affected the neuronal differentiation and cell viability, we attempted to confirm the inhibitory effect of RU 486 (a GR antagonist) on the CORT action on **eNSPCs**. However, we could not observe inhibition by RU 486 because solo-application of RU 486 induced cell death in our **eNSPC** cultures (data not shown). Therefore we next examined the effect of DEX, which has approximately 30 times higher affinity for GR (but not MR) than CORT, on the phenotype of **eNSPCs** (Kawata, 1995). As expected, DEX mimicked the CORT action at lower dose (0.1-10 μ M) than CORT, suggesting that the activation of GR is primarily involved in the decreased differentiation and survival of **eNSPC** under GCs exposure. **Another possible target of GCs is membrane GR (mGR). This plasma membrane-localized receptor activates the downstream cascade of G protein-coupled receptors and mediates the rapid non-genomic action of GCs (Tasker et al. 2006). Previous study suggests that mGR signaling rapidly affects neuronal function through endocytosis of cell surface receptors, cytoskeletal rearrangements and so on (Yang et al. 2013). Although long-lasting effect of mGR signaling remains unclear, it might be involved in the cellular response of NSPC via the modulation of transcriptional factor.**

Although a number of studies has demonstrated that evoked ERK and/or PI3K/Akt signaling by exogenously applied factors promote neural differentiation and survival of **NSPC**, it is still incompletely understood the contribution of basal ERK and PI3K/Akt signaling activity on the differentiation and cell survival (Ishii et al., 2010; Liu et al., 2014; Nguyen et al., 2009; Qiao et al., 2012). Here, we found a significant elevation of pERK and pAkt in **eNSPCs** during the differentiation stage and the **increase** of basal ERK and PI3K/Akt signaling **activity** having a pivotal role for survival and differentiation of **eNSPCs**. An ERK signaling inhibitor, U0126, decreased expression of GAD65/67 and cell survival at 7 days (but not 3 days) on the differentiation phase. These results were consistent with the temporal change of ERK activity that was significantly increased during 5-7 days, implying that ERK activation is required for **NSPC** differentiation into GABAergic neurons and their survival in the late phase of differentiation. In contrast, upregulation of pAkt occurred at early phase of differentiation (3-5 days). An Akt

signaling inhibitor, LY294002, caused decrease of neuronal proteins and cell viability at 3 and 7 days, implying the importance of PI3K/Akt signaling during relatively early phase of the differentiation. The consistency of the effective dose of CORT (10 μ M) in our culture experiments also supported that the inhibition of ERK and Akt signaling was involved in CORT action.

Previous studies including ours have reported that GCs exposure impaired factor-induced stimulation of ERK signaling and suppressed synaptic, neural, and glial maturation (Heberden et al., 2013; Kumamaru et al., 2011). In the present study, our results propose that the inhibition of PI3K/Akt signaling is also involved in the negative action of GCs on neuronal differentiation and cell survival in eNSPCs. Although the molecular mechanism of the inhibitory action of CORT on these signaling pathways is still unclear, it is possible that CORT specifically perturbed activation of the pathways at the differentiation stage as CORT did not affect the proliferation of eNSPC which depends on the ERK and PI3K/Akt activation induced by EGF and bFGF. It also supported this idea that inhibition of pERK2 and pAkt by 10 μ M CORT was not observed at 1 day after differentiation (data not shown). Thus, to reveal the molecular mechanism behind reduced signaling caused by GCs, further investigation on increased basal activity of signaling during differentiation is need.

IGF-I plays roles in normal brain development through promoting NSPC proliferation, survival, neural differentiation, and synaptogenesis through binding to type I IGF receptor, and subsequent activation of PI3K/Akt and ERK signaling (Ye and D'Ercole. 2006; Bondy and Cheng. 2004). Recent studies have reported a potential use of IGF-I as a pharmacological treatment for neurodevelopmental diseases such as Rett syndrome (Marchetto et al. 2010; Castro et al. 2014). IGF-I treatment restored synaptic deficiency and improved behavioral abnormalities in a Rett syndrome mouse model in which the gene encoding transcriptional factor MeCP2 is mutated (Castro et al. 2014). Our results also showed that IGF-I improved the suppression of PI3K/Akt signaling caused by CORT exposure and impairment of neural differentiation and synaptogenesis. Thus, IGF-I might be useful for the future treatment of neurodevelopmental disturbance caused by prenatal stress.

Besides the neurogenesis, GCs exposure affected differentiation and maturation of astrocytes. GFAP (an intermediate filament of astrocytes) increases during cell development, which is essential for the proper integrity of brain white matter, myelination, and formation of blood-brain barrier (Gomes et al., 1999). Furthermore, O'Callaghan reported that CORT administration to adult rats resulted in decreased GFAP expression throughout the brain (O'Callaghan et al., 1989). Importantly, repeated administration of synthetic GC in pregnant ewes caused the decrease of GFAP-positive area of the corpus callosum, and delay in capillary tight junction maturation in the fetal sheep brain (Huang et al., 2001). Here, we showed that CORT/DEX exposure decreased GFAP expression at 3 and 7 days after the differentiation initiation. Unlike the inhibition of neural differentiation, reduced GFAP expression was induced by 0.1-10 μ M CORT at 3 days. In addition, unlike the suppression of GFAP expression by the Akt inhibitor, CORT (1-10 μ M)-induced GFAP reduction was remained until 7 days. Considering this inconsistency and the existence of glucocorticoid response element in the promoter region of the GFAP gene, there would be other mechanism as well as the suppression of Akt signaling, such as a direct transcriptional regulation of GFAP gene via GR function (Heberden et al., 2013).

In summary, our results demonstrated that chronic CORT exposure suppressed the progress of neuronal differentiation and increased cell vulnerability of eNSPC. Further, we found the inhibitory effect of CORT on ERK and PI3K/Akt signaling during the eNSPC differentiation as a possible mechanism underlying the adverse effect of CORT. Our *in vitro* model would be beneficial to further investigate the adverse effect of GCs stress on the early brain development.

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Figure Legends

Fig.1. GC exposure did not affect the proliferation of eNSPCs.

(A) **eNSPCs** were exposed to CORT (0.001-10 μ M) for 3 days proliferation phase. Cell viability at 3 days proliferation phase was examined by MTT assay. (n=6-12 in each condition). (B) Cell viability was assessed after proliferation phase with or without DEX treatment by MTT assay (n=6-12 in each conditions). (C) The changes in marker protein expression during differentiation phase are shown. After proliferation phase, the differentiation of **eNSPCs** was initiated by removing mitogens from culture medium. Marker proteins (SOX2, DCX, syntaxin, GAD65/67, and GFAP) at 0-7 days after the initiation of differentiation were determined by Western Blotting (WB, *left panel*). β -Actin is shown as control protein. Quantification of each band intensity is shown in the *right panel* (n=4 in each condition). *** $P < 0.001$, * $P < 0.05$ vs. 0 day. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test.

Fig.2. Impaired differentiation and reduced cell viability of eNSPCs were caused by CORT exposure.

eNSPCs were exposed to CORT (0.1-10 μ M) for 3 days proliferation phase and subsequent 7 days differentiation period. (A)(B) Expression levels of marker proteins (SOX2, DCX, syntaxin, GAD65/67, and GFAP) at 3 (A) or 7 days (B) after the induction of differentiation (WB, *left panel*). Quantification graphs are also shown in *right* (n=5-9 in each condition). (C) The cell viability at 3 (*left*) or 7 days (*right*) was assessed by MTT assay (n=6 in each condition). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. without CORT. One-way ANOVA (Bonferroni *post-hoc* test).

Fig.3. DEX treatment impaired the differentiation of eNSPCs and increased cell vulnerability of eNSPCs.

eNSPCs were exposed to DEX (0.1-10 μ M) throughout the proliferation phase and subsequent differentiation phase. (A)(B) Expression levels of marker proteins (including DCX, syntaxin, and GFAP) at 3 (A) or 7 days (B) after the differentiation initiation were assessed by WB (*left panel*).

Quantification of each band is shown in the *right* (n=5-6 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. without DEX. One-way ANOVA (Bonferroni *post-hoc* test). (C) Cell viability at 3 (*left*) or 7 days (*right*) was determined by MTT assay (n=6 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. without DEX. One-way ANOVA (Bonferroni *post-hoc* test).

Fig.4. CORT suppressed activation of ERK and Akt signaling during differentiation of eNSPCs.

(A) Phosphorylated ERK1/2 and Akt at 1-7 days after the differentiation initiation determined by WB with anti-pERK1/2 or anti-pAkt antibody. After stripping the membrane, re-blotting with anti-total ERK1/2 or Akt antibody was performed (*left panel*). *Right*; Quantified data (n=3-4 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. 1 day. (B)(C) pERK1/2 and pAkt levels with or without CORT (0.1-10 μ M) at 3 (B) or 7 days (C). Total-ERK1/2 and Akt were also indicated. Quantified data are shown the in *right* (n=3-9 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. without CORT. One-way ANOVA (Bonferroni *post-hoc* test).

Fig.5. Involvement of ERK and Akt signaling in the differentiation and survival of eNSPCs.

Treatment with U0126 (10 μ M) or LY294002 (10 μ M) was performed during 1-7 days after the start of differentiation. (A)(B) Levels of pERK1/2 and marker proteins at 3 (A) or 7 days (B) after the induction of differentiation. The membrane was stripped and reprobed with anti-total ERK antibody. Quantification of each band is also shown in the *right* (n=4-5 in each conditions). *** P<0.001 vs. without U0126. (C)(D) Levels of pAkt and marker proteins at 3 (C) or 7 days (D). The membrane was stripped for reprobed with anti-total Akt antibody. Quantification is displayed in the *right panel* (n=3-5 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. without LY294002. (E) Cell viability after U0126 or LY294002 treatment at 3 (*left*) or 7 days (*right*) (n=6 in each conditions). *** P<0.001, ** P<0.01 vs. control. Student's *t*-test.

Fig.6. IGF-I restored the impairment of eNSPC differentiation caused by CORT

Treatment of CORT (10 μ M) was performed as described in Fig.2. IGF-I (100 ng/ml) was applied at 1 day after the initiation of differentiation. (A) Levels of phosphorylated ERK1/2, Akt and these total forms at 7 days after differentiation initiation were determined by WB. Quantification of each band is shown in the *right* (n=3-4 in each conditions). *** P<0.001, ** P<0.01, * P<0.05 vs. - CORT - IGF-I. †† P<0.01. (B) Levels of DCX, syntaxin, and GFAP at 7 days after differentiation initiation were determined by WB. Quantification is displayed in the *right panel* (n=4 in each conditions). *** P<0.001, * P<0.05 vs. - CORT - IGF-I. ††† P<0.001, †† P<0.01. Two-way ANOVA (Bonferroni *post-hoc* test).

Figure 1

A **B** **C**

Figure 2**A**

3 days after differentiation

B

7 days after differentiation

C

3 days after differentiation

7 days after differentiation

Figure 3

A

3 days after differentiation

B

7 days after differentiation

C

3 days after differentiation

7 days after differentiation

Figure 4

A

B

C

Figure 5**A**

3 days after differentiation

B

7 days after differentiation

C

3 days after differentiation

D

7 days after differentiation

E

3 days after differentiation

7 days after differentiation

Figure 6

A

B