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Possible Involvement of PI3-K/Akt-Dependent GSK-3β Signaling in Proliferation of Neural Progenitor Cells After Hypoxic Exposure

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Abstract

We previously demonstrated that proliferation of endogenous neural progenitor cells is enhanced by cerebral ischemia and that phosphatidylinositol 3-kinase (PI3-K)/Akt-dependent glycogen synthase kinase (GSK)-3β signaling is involved in ischemia-induced neurogenesis. It is important to learn more about the regulation of proliferation and differentiation of neural progenitor cells under ischemic conditions, as such knowledge that may serve as the basis for the development of new therapeutic approaches for stroke. However, it remains to be addressed whether a change in that signaling pathway is induced in neural progenitor cells. We prepared neural progenitor cells by using the neurosphere method and conducted experiments to determine the relative contributions of the PI3-K/Akt-dependent GSK-3β signaling pathway to the proliferation and differentiation of neural progenitor cells under the hypoxic condition in vitro. We showed that hypoxic exposure induced the proliferation of neural progenitor cells. This proliferation was accompanied by phosphorylation of Akt and GSK-3β at its Ser9. Furthermore, treatment with a PI3-K inhibitor decreased the hypoxia-induced phosphorylation of GSK-3β and proliferation of neural progenitor cells. Furthermore, hypoxic exposure enhanced the differentiation of neural progenitor cells, and this increased differentiation was not affected by treatment with the PI3-K inhibitor. Although the expression of NeuroD1 mRNA during cell differentiation was also enhanced by hypoxic exposure, this increased expression was not affected by treatment with the PI3-K inhibitor. Our findings suggest that the PI3K/Akt-dependent GSK-3β signaling pathway was involved in the proliferation of neural progenitor cells under a pathologic condition, such as hypoxia and/or cerebral ischemia in vivo.

Keywords Cerebral ischemia · Neural progenitor cell · Neurogenesis · GSK-3β · PI3-K/Akt · Hypoxia

Abbreviations

GSK	Glycogen synthase kinase
PI3-K	Phosphatidylinositol 3-kinase
SGZ	Sub-granular zone

Introduction

Stroke is a major cause of disability and mortality worldwide. Although stroke is categorized into ischemic and hemorrhagic types of stroke, the majority of stroke cases fall into the ischemic category. Because of the very narrow therapeutic time window, current therapeutic strategies for stroke, such as thrombolysis, are far from optimal. Furthermore, stroke survivors suffer severe aftereffects, including motor dysfunction and cognitive impairment. Therefore, only rehabilitation appears to be effective to promote functional recovery in stroke patients.

Neurogenesis occurs in two restricted regions of the brain, i.e., the subventricular zone of the lateral ventricles and subgranular zone (SGZ) of hippocampal dentate gyrus, and it is accelerated after cerebral ischemia [1, 2]. It has been suggested that neurogenesis is closely associated with neurological functions such as spatial learning and memory and that it is affected by various factors including age, drugs, and the environment. Therefore, cerebral ischemia-induced neurogenesis has been expected to repair brain injuries by replacing lost cells after cerebral ischemia. However, it seems that the majority of newly generated endogenous neurons after cerebral ischemia fail to survive [3, 4]. Although acceleration of endogenous neurogenesis in the brain has the potential to become a new therapeutic approach for stroke, the pathophysiological alterations underlying cerebral ischemia-induced

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neurogenesis remain to be elucidated. In this sense, we previously demonstrated that the number of NeuroD1-positive cells is increased on day 7 in the dentate gyrus of the hippocampus after cerebral ischemia [5]. NeuroD1 is a proneural basic helix-loop-helix (bHLH) transcription factor and promotes pre/mature cell-cycle exit and differentiation into neural progenitor cells [6, 7]. Furthermore, we recently reported that enhanced expression of NeuroD1 after cerebral ischemia is associated with the phosphatidylinositol 3-kinase (PI3-K)/Akt-dependent glycogen synthase kinase (GSK)-3β signaling pathway [5]. However, it has not been addressed whether a change in this signaling pathway is induced in neural progenitor cells. It is important to determine in detail the regulation of the proliferation and differentiation of neural progenitor cells under ischemic conditions for a better understanding of the pathophysiology of stroke.

In this study, we prepared neural progenitor cells by using the neurosphere method and conducted experiments to determine the relative contributions of the PI3K/Akt-dependent GSK-3β signaling pathway to proliferation and differentiation of neural stem/progenitor cells under the hypoxic condition in vitro.

Materials and Methods

Neural Stem/Progenitor Cell Cultures

Neural stem/progenitor cells were prepared from gestational day-14 fetal rats by the method described previously [8]. Cells were seeded at a density of 50,000 cells/cm^2 into non-treated flasks containing DMEM/F12 supplemented with N-2 Max supplement (R&D Systems, Inc., Mckinley Place, MN, USA), 20 ng/ml epidermal growth factor (EGF; Peprotech-TanyTechnoGene Ltd., Rehovot, Israel), and 20 ng/ml basic fibroblast growth factor (b-FGF; growth medium; Peprotech). Neural stem/progenitor cells were grown in culture as free-floating neurospheres in a 37 °C, 5% CO_2 incubator. The rats had free access to food and water according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Guidance for Experimental Animal Care issued by the Prime Minister's Office of Japan. The study was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Sciences.

Immunohistochemistry

For immunostaining, floating cultured neurospheres were incubated on MAS coating micro slide glass (Matsunami Glass Ind., Ltd.) for 30 min at 25 °C. After having been washed, the attached neurospheres were immunestained as described previously [9]. In some experiments, the cells were immunestained after differentiation. The primary antibodies used were mouse monoclonal anti-Ki67 (BD Pharmingen), rabbit polyclonal anti-musashi-1 (Cell Signaling), goat polyclonal anti-DCX (Santa Cruz Biotechnology Inc.), mouse monoclonal anti-nestin (Abcam), rabbit polyclonal anti-Sox2 (Abcam), mouse monoclonal anti-glial fibrillary acidic protein (GFAP; BD biosciences), rabbit monoclonal anti-β3-tubulin (Cell Signaling), mouse monoclonal anti-CNPase (Abcam), and mouse monoclonal anti-vimentin (Sigma-Aldrich) antibodies. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories, PA, USA), FITC-conjugated goat anti-mouse IgG (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), and Cy3-conjugated anti-goat IgG (Jackson Immuno-Research Laboratories). Fluorescent images of cells were captured by a CCD camera (DP50) mounted on an Olympus fluorescence microscope (BX-52, Olympus, Tokyo, Japan) equipped with a mercury arc lamp.

Hypoxic Exposure

Neurospheres were cultured for 7 days and dissociated into single cells by using Accutase (Invitrogen Co., Carlsbad, CA, USA). The cell density of neural stem/progenitor cells was adjusted to 1×10^6 well for Western blotting and qRT-PCR, to 1×10^5 cells/well for immunocytochemistry and neurosphere formation assay, and to 5×10^4 cells/well for cell proliferation assays prior to the treatments. For cultures in hypoxic environments, cells were placed in a hypoxic chamber, which was initial flushed with a mixture of 95% N_2 and 5% CO_2, within a humidified modular incubator 37 °C. The oxygen concentration in the chamber was maintained at 5% with a residual gas mixture of 5% CO_2 and balanced nitrogen for 24, 48, or 96 h at 37 °C. For cultures in normoxic environments, cells as a matched control group were cultured in 95% atmospheric air and 5% CO_2 for the same times as cells in hypoxic condition at 37 °C. Neurospheres were treated simultaneously with LY294002 (1 μM; Cayman chemical, Ann Arbor, MI, USA), an inhibitor of PI3-K and hypoxic exposure.

Cell Proliferation and Neurosphere Formation Assays

Cell proliferation was assessed by performing the sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) assay (Sigma-Aldrich, St. Louis, MO, USA) and neurosphere formation assay. XTT assay has been widely used to measure for cell proliferation by detecting redox potential of cells [10, 11]. An aliquot (250 μL) of cell suspensions was seeded into non-treated 24-well plates and cultured for 48 h under the hypoxic condition. XTT-PMS solution (125 μL; 1 mg/ml XTT and 1.54 mg/ml PMS) was added to medium containing neural stem/progenitor cells. After 2 h of incubation at 37 °C, the absorbance was measured with a microplate reader at 450 nm. The relative cell proliferation was expressed as the

ratio of the absorbance of each group against the normoxia group. In the neurosphere formation assay, dissociated cells were seeded at 1×10^5 cells/well into non-treated 6-well plates and cultured for 48 h under the normoxic or hypoxic condition. Five phase contrast images (1800 × 1350 μm) were taken per well by an Olympus microscope (CKX-53; Olympus). Diameter of all neurospheres in the images was measured. Furthermore, the number of neurospheres with a diameter of 40 μm or more was counted.

Western Immunoblotting

Dissociated cells were seeded at a density of 1×10^6 cells/well into non-treated 6-well plates and cultured for 24 or 48 h under the normoxic or hypoxic condition. After incubation, the cells were collected and lysed in lysis buffer containing 50 mM Tris, 1 mM EDTA, 0.1% deoxycholate, 1% TritonX-100 along with a cocktail of protease and phosphatase inhibitors (Roche Diagnostics Co.) at 4 °C. Nuclear fraction was isolated using Lysopure Nuclear and Cytoplasmic Extractor kit (Wako) according to manufacturer's instrument. Protein concentrations were determined by using the BCA protein Assay (Thermo Fisher Scientific Waltham, MA, USA). Western blotting was performed according to standard protocols. The following primary antibodies were used: rabbit monoclonal anti-phospho-GSK-3β (Ser 9; Cell Signaling Technology Inc., Danvers, MA, USA), rabbit monoclonal anti-GSK-3β (Cell Signaling), rabbit monoclonal anti-phospho-Akt (Cell Signaling), rabbit monoclonal anti-Akt (Cell Signaling), rabbit monoclonal anti-active-β-catenin (Cell Signaling), rabbit monoclonal anti-β-catenin (Cell Signaling), and mouse monoclonal anti-β-actin (Sigma-Aldrich) antibody. Subsequently, the protein blots were washed and incubated with the appropriate secondary antibodies. Bound antibodies were detected by the enhanced chemiluminescence method (Amersham Biosciences Inc., Piscataway, NJ, USA). Quantification was performed using computerized densitometry (Luminograph II, ATTO Co., Tokyo, Japan) and an image analyzer (CS Analyzer, ATTO Co., Tokyo, Japan).

Cell Differentiation Assay

Dissociated cells were seeded at a density of 5×10^4 cells/well into poly-D-lysine-coated 6-well plates containing DMEM/F12 supplemented with 2% B27 supplement and 1% fetal bovine serum and allowed to differentiate for 96 h under the normoxic or hypoxic condition. Immunocytochemistry was performed to analyze cell differentiation. Cells were first fixed in 4% PFA for 20 min and then washed three times with PBS. Next, they were incubated overnight with the desired primary antibody at 4 °C and subsequently with the corresponding secondary antibody for 1 h at 25 °C. Nuclear staining was achieved with Hoechst33342 (10 min at 25 °C) to count the number of total cells. The following primary antibodies were used: rabbit monoclonal anti-β3-tubulin antibody (Cell Signaling). Secondary antibody was the following: Alexa Fluor 594-labeled goat anti-rabbit IgG antibodies. Fluorescence was detected by using an Olympus fluorescence microscope (IX-71; Olympus). Eight images (435 × 330 μm) were randomly taken per well and loaded into the MetaMorph software program (Molecular Devices, Downingtown, PA). Based on background fluorescence and the size of their nucleus, Hoechst33342-stained cells were counted by use of the MetaMorph software program in eight images per experiment and the ratio of β3-tubulin- or vimentin-positive cells to Hoechst33342-stained cells was calculated. The data obtained from four independent experiments.

qRT-PCR

Dissociated cells were seeded at 1×10^6 cells/well into poly-D-lysine-coated 6-well plates and allowed to differentiate for 48 h under the normoxic or hypoxic condition. After incubation, total RNA of differentiated cells was isolated by using an RNeasy mini kit (QIAGEN N.V., Venlo, Netherlands), and cDNAs were synthesized from 500 ng of total RNAs by using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO CO., LTD., Tokyo, Japan). qRT-PCR was performed using THUNDERBIRD® SYBR qPCR Mix (TOYOBO CO., LTD.) on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Data were normalized to the β-actin mRNA expression and analyzed by the $2^{-\Delta\Delta Ct}$ method. Primers used in the present study were as follows: GFAP—forward, 5′-CCAG ATCCGAGAACCAGCC-3′; reverse, 5′-CCGC ATCTCCACCGTCTTTA-3′. NeuroD1—forward, 5′-GAAC ACGAGGCAGACAAGAA-3′; reverse, 5′-TCAT CTTCATCCTCCTCCTCTC-3′. β-actin—forward, 5′-TGCT ATGTTGCCCTAGACTTCG-3′; reverse, 5′-GTTG GCATAGAGGTCTTTACGG-3′.

Statistical Analysis

The results were expressed as the means ± standard error of the mean (SEM). Differences between two groups were evaluated statistically by use of the unpaired Student's *t* test. Statistical comparison among multiple groups was evaluated by analysis of variance (ANOVA), followed by Bonferroni test as a post hoc test. *P* values of less than 0.05 were considered significant.

Results

Prepared Cells Expressed the Properties of Neural Stem Cells and Hypoxic Exposure Enhanced the Proliferation of Neural Stem/Progenitor Cells

We initially examined whether the prepared cells, which were collected by the neurosphere method, expressed markers for proliferation, neural stem cells, and neuronal progenitor cells on day 7 when cultured in vitro. Proliferation marker Ki67 (Fig. 1a, b) and neural stem markers nestin (Fig. 1a, c, d), Sox2 (Fig. 1b), and musashi1 (Fig. 1c) were expressed in the prepared neurospheres, although the expression of neural stem marker DCX was weak (Fig. 1d). We also confirmed the expression of markers for neurons (β3-tubulin), astrocytes (GFAP), and oligodendrocytes (CNPase) in the cells at 7 days after the induction of differentiation (Fig. 1e). These results reveal the ability of the prepared cells in this study to express the properties of self-renewal and multipotency. The diameter of the neurospheres was increased by 48 h of hypoxic exposure (Fig. 2a). Cell proliferation assessed by the XTT assay was also significantly enhanced after 48 h of hypoxic exposure as compared with that after normoxic exposure (Fig. 2b, unpaired *t* test, $t = 8.889$, $P = 0.0001$).

Hypoxic Exposure Increased Phosphorylation of Akt and GSK-3β and the Level of β-Catenin in Nuclear Fraction of Neural Stem/Progenitor Cells

Phosphorylation of Akt at its Ser473 results in the activated form of Akt, whereas phosphorylation of GSK-3β at its Ser9 by phosphorylated Akt inactivates GSK-3β. Next, we examined changes in the levels of activated Akt and inactivated GSK-3β of neural stem/progenitor cells after hypoxic exposure. The levels of phosphorylated Akt after 24 and 48 h of hypoxic exposure were increased as compared with those of the phosphorylated forms under the normoxic condition (Fig. 2c, unpaired *t* test, $t = 8.004$, $P = 0.0002$ and Fig. 2d, unpaired *t* test, $t = 3.216$, $P = 0.018$). The phosphorylation of GSK-3β was also enhanced after 24 or 48 h of hypoxia (Fig. 2e, unpaired *t* test, $t = 5.930$, $P = 0.00103$), although that after 48 h of hypoxia was not statistically significant difference (Fig. 2f, unpaired *t* test, $t = 1.7819$, $P = 0.125$). It is known that phosphorylation of β-catenin by GSK-3β is involved in the degradation of β-catenin by the ubiquitin-proteasome system. In contrast, non-phosphorylated β-catenin is translocated to the nucleus. Therefore, we next examined the level of β-catenin in nuclear fraction. The level of β-catenin in nuclear fraction after 24 h of hypoxic exposure was increased as compared with that after normoxic exposure (Fig. 2g, unpaired *t* test, $t = 2.668$, $P = 0.0371$). The level of β-catenin in nuclear fraction after 48 h of hypoxic exposure tended to increase as compared with that after normoxic exposure (Fig. 2h, unpaired *t* test, $t = 1.781$, $P = 0.1251$).

The Proliferation of Neural Stem/Progenitor Cells Was Enhanced by Hypoxic Exposure via the PI3K/Akt Pathway

We next determined the effect of LY294002, an inhibitor of PI3-K, on the proliferation of neural stem/progenitor cells after 48 h of hypoxic exposure. Treatment with LY294002 attenuated the enhanced proliferation of neural stem/progenitor cells after 48 h of hypoxic exposure assessed by XTT assay (Fig. 3a, Bonferroni's post hoc test, $P = 0.0137$). Since the treatment with 1 μM LY294002 decreased the proliferation of neural stem/progenitor cells after hypoxic exposure without causing changes in cell proliferation after normoxic exposure (Fig. 3a), this concentration of LY294002 was used in the subsequent study. Furthermore, the average diameters of neurospheres were increased by 48 h of hypoxic exposure (Fig. 3b, c, Bonferroni's post hoc test, $P = 0.0016$) and the increased diameters after hypoxic exposure were not affected by treatment with LY294002 (Fig. 3b, c, Bonferroni's post hoc test, $P = 0.0016$). As the average diameter of neurospheres under normoxic exposure was 45.7 ± 1.1 μm (Fig. 3c), we next measured the number of neurospheres with a diameter of 40 μm or more in the field. The results demonstrated that the number of neurospheres with a diameter of 40 μm or more was significantly increased after hypoxic exposure (Fig. 3d, Bonferroni's post hoc test, $P = 0.0284$). This increased number was attenuated by treatment with LY294002 (Fig. 3d, Bonferroni's post hoc test, $P = 0.0319$).

We next determined the effects of LY294002 on the phosphorylation of Akt and GSK-3β after hypoxic exposure. The increased phosphorylation of Akt after 24 or 48 h of hypoxic exposure was inhibited by the treatment with LY294002 (Fig. 4a, Bonferroni's post hoc test, $P = 0.0036$ H versus H + LY294002 and Fig. 4b, $P = 0.0006$ H versus H + LY294002). Also, the marked increase in phosphorylation of GSK-3β after 24 h of hypoxic exposure was suppressed by the treatment with the inhibitor (Fig. 4c, Bonferroni's post hoc test, $P = 0.0476$ N versus H and $P = 0.0024$ H versus H + LY294002). The increased level of nuclear β-catenin after 24 h of hypoxic exposure was inhibited by the treatment with LY294002 (Fig. 4e, Bonferroni's post hoc test, $P = 0.0008$ N versus H and $P = 0.0028$ H versus H + LY294002). The levels of β-catenin in nuclear fraction after 48 h of hypoxic or normoxic exposure were not altered regardless of treatment with LY294002 (Fig. 4f).

Differentiation of Neural Stem/Progenitor Cell Was Promoted by Hypoxia, but Not Through the PI3K/Akt Pathway

We further examined the effects of inhibition of PI3-K on the differentiation of neural stem/progenitor cells after 96 h of hypoxic exposure. In the differentiation assay, the total number of cells was not affected by hypoxic exposure regardless of

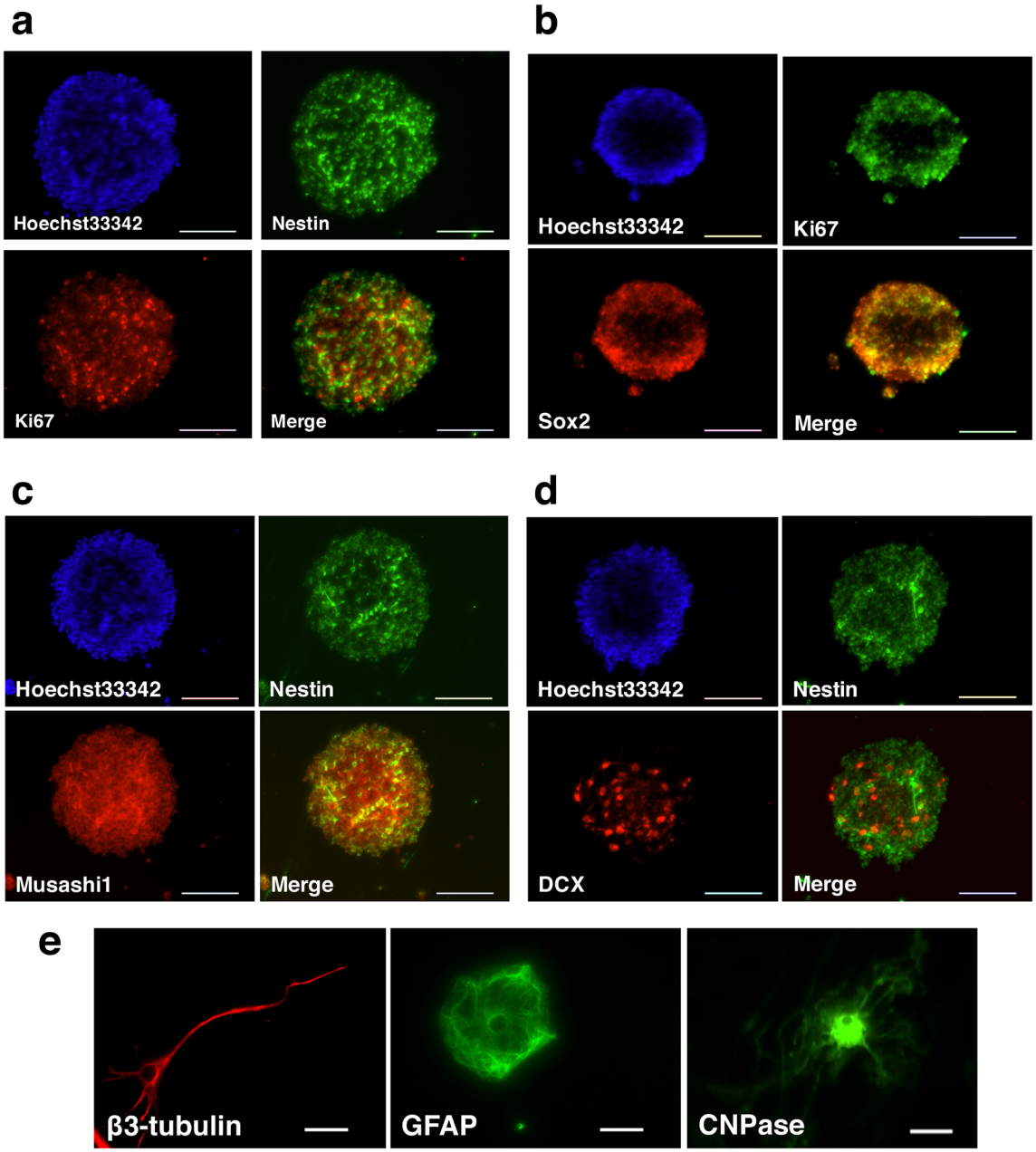

Fig. 1 Characterization of cells in neurospheres. The results of triple staining with Hoechst 33342 (**a**–**d**), anti-nestin (**a**, **c**, and **d**), anti-Ki67 (**a** and **b**), anti-sox2 (**b**), musashi-1 (**c**), and anti-DCX (**d**) of cells in neurospheres on day 7 are shown. **e** Immunostaining results for β3-tubulin for neurons, GFAP for astrocytes, and CNPase for oligodendrocytes in cells after differentiation are shown. Scale bar, 100 μm (**a**–**d**), 50 μm (**e**)

treatment or not with LY294002 (Fig. 5b). To determine the effects of hypoxic exposure on the differentiation to neurons and astrocytes, we used anti-β3-tubulin and anti-vimentin antibodies as a marker of neuron and astrocyte, respectively (Fig. 5a). The ratio of β3-tubulin-positive cells to Hoechst33342-positive cells was significantly increased after hypoxic exposure (Fig. 5a, c, Bonferroni's post hoc test, $P = 0.0402$ N versus H). In contrast, the ratio of vimentin-positive cells to Hoechst33342-positive cells was decreased after hypoxic exposure (Fig. 5a, d, Bonferroni's post hoc test, $P = 0.0446$ N versus H). The ratios of β3-tubulin-positive cells and vimentin-positive cells to Hoechst33342-positive cells after 96 h of hypoxic exposure were not affected by the treatment with LY294002, respectively (Fig. 5c, Bonferroni's post hoc test, $P > 0.05$ H versus H + LY294002 and $P = 0.0332$ N + LY294002 versus H + LY294002 and Fig. 5d, $P > 0.05$ H versus H + LY294002 and $P = 0.0399$ N + LY294002 versus H + LY294002).

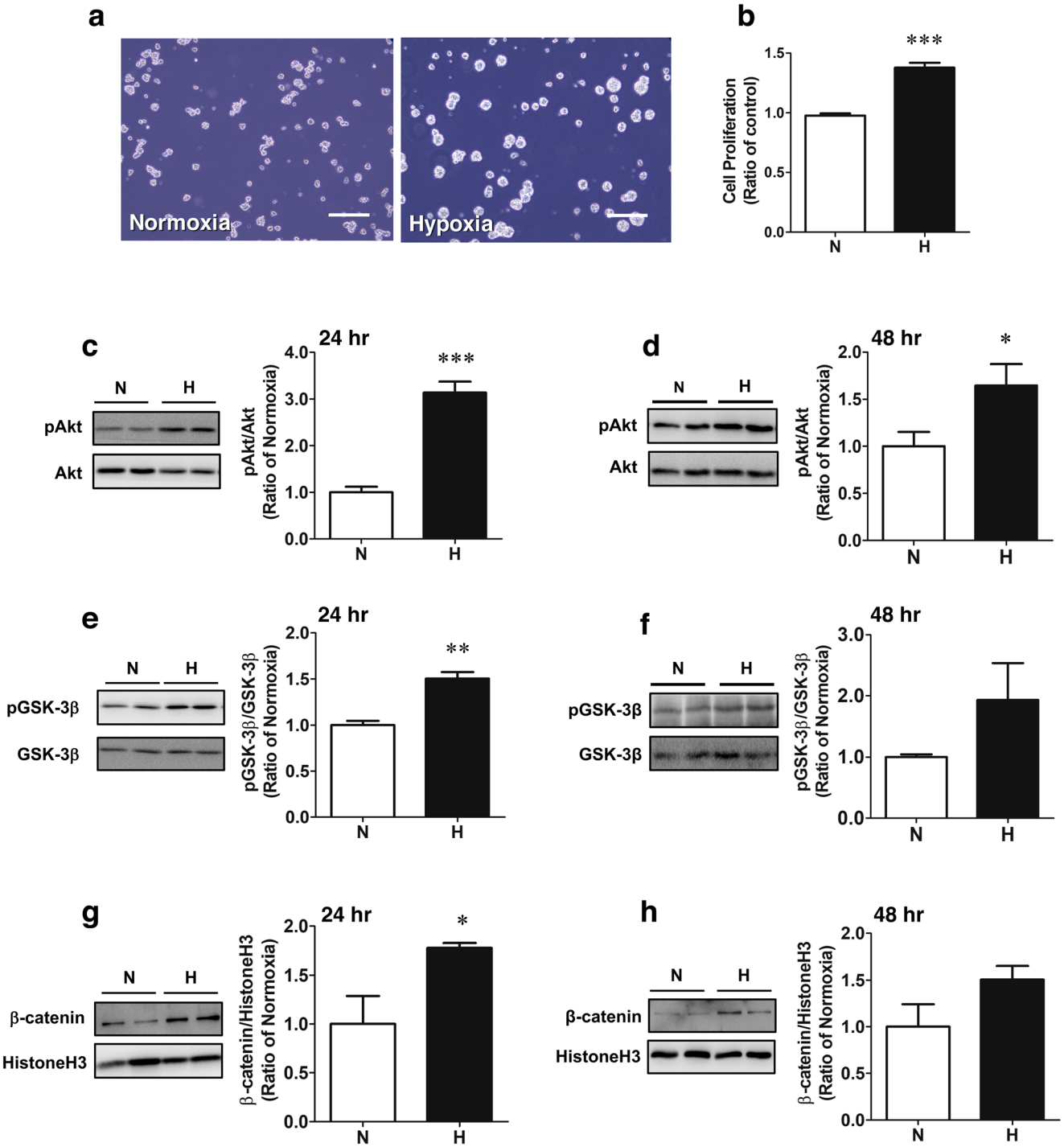

Fig. 2 Characterization of neurospheres. **a** Changes in diameter of neurospheres after hypoxic exposure are shown. Scale bar, 200 μm. **b** Cell proliferation assessed by the XTT assay after hypoxic (H) exposure was enhanced compared with that after normoxic (N) exposure. Values are presented as means ± SEM, $n = 5$ independent experiments. ***Significant difference from the normoxic group ($P < 0.001$). Changes in the levels of phosphorylated Akt and GSK-3β and non-phosphorylated β-catenin after hypoxic exposure. **c** and **d** Changes in the phosphorylation of Akt and total levels of Akt after normoxic (N) and hypoxic (H) exposure for 24 (**c**) or 48 (**d**) hours. **e** and **f** Changes in the phosphorylation of GSK-3β at its Ser9 and total levels of GSK-3β after normoxic (N) and hypoxic (H) exposure for 24 (**e**) or 48 (**f**) hours. **g** and **h** Changes in the levels of β-catenin in the nuclear after normoxic (N) and hypoxic (H) exposure for 24 (**g**) or 48 (**h**) hours. Results are expressed as the mean ratio of the hypoxic group to the normoxic group ± SEM ($n = 4$ independent experiments). *Significant difference from the normoxic group ($P < 0.05$). **Significant difference from the normoxic group ($P < 0.01$). ***Significant difference from the normoxic group ($P < 0.001$)

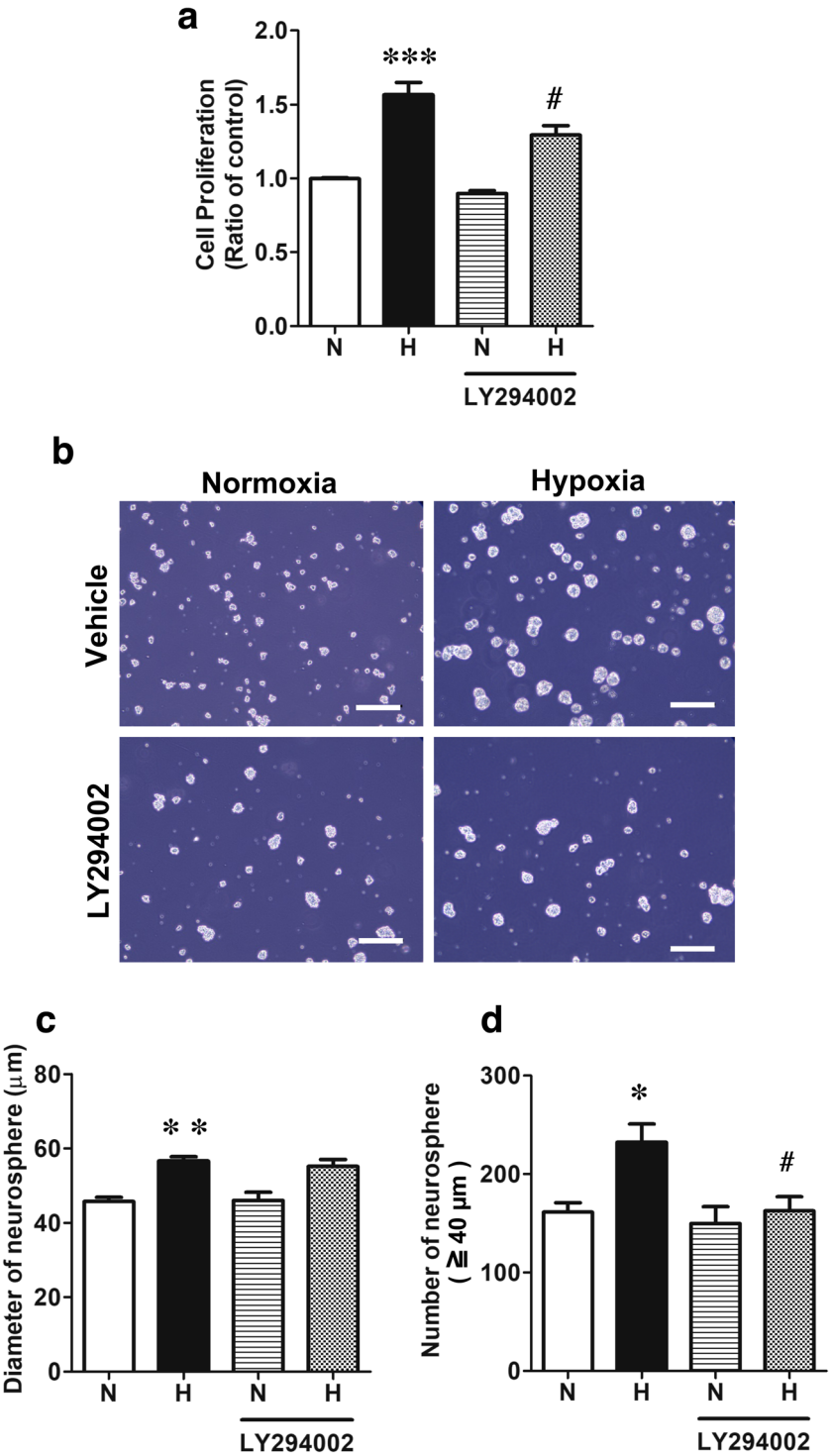

Fig. 3 Effect of LY294002, an inhibitor of PI3-K, on the proliferation of neural stem/progenitor cells. **a** Effects of LY294002 on the proliferation of neural stem/progenitor cells after 48 h of normoxic (N) and hypoxic (H) exposure with or without LY294002 treatment (1 μM). **b** Photomicrographs of neurospheres after normoxic or hypoxic exposure with 1 μM LY294002 or vehicle (0.1% DMSO) treatment. Scale bar, 200 μm. **c** Effect of LY294002 on the diameter of neurospheres after normoxic (N) or hypoxic (H) exposure. **d** Effect of LY294002 on the number of neurospheres with a diameter of 40 μm or more in the field after normoxic (N) or hypoxic (H) exposure. Results are expressed as the mean ± SEM ($n = 5$ independent experiments). *Significant difference from the normoxic group ($P < 0.05$). **Significant difference from the normoxic group ($P < 0.01$). ***Significant difference from the normoxic group ($P < 0.001$). #Significant difference from the LY294002-untreated hypoxic group ($P < 0.05$)

The Expression of NeuroD1 mRNA Was Enhanced by Hypoxic Exposure During Cell Differentiation

We next investigated whether the inhibition of PI3-K would affect the mRNA expression of NeuroD1, which is a proneural basic helix-loop-helix transcription factor, during hypoxia-induced cell differentiation. As it is expressed only in an extremely short period at the time of differentiation [12, 13], we examined effects of hypoxic exposure on expression of NeuroD1 mRNA during cell differentiation with or without LY294002 treatment. The expression of NeuroD1 mRNA was enhanced by hypoxic exposure during cell differentiation (Fig. 5e, Bonferroni's post hoc test, $P < 0.05$ N versus H), and this increased expression was not affected by treatment with

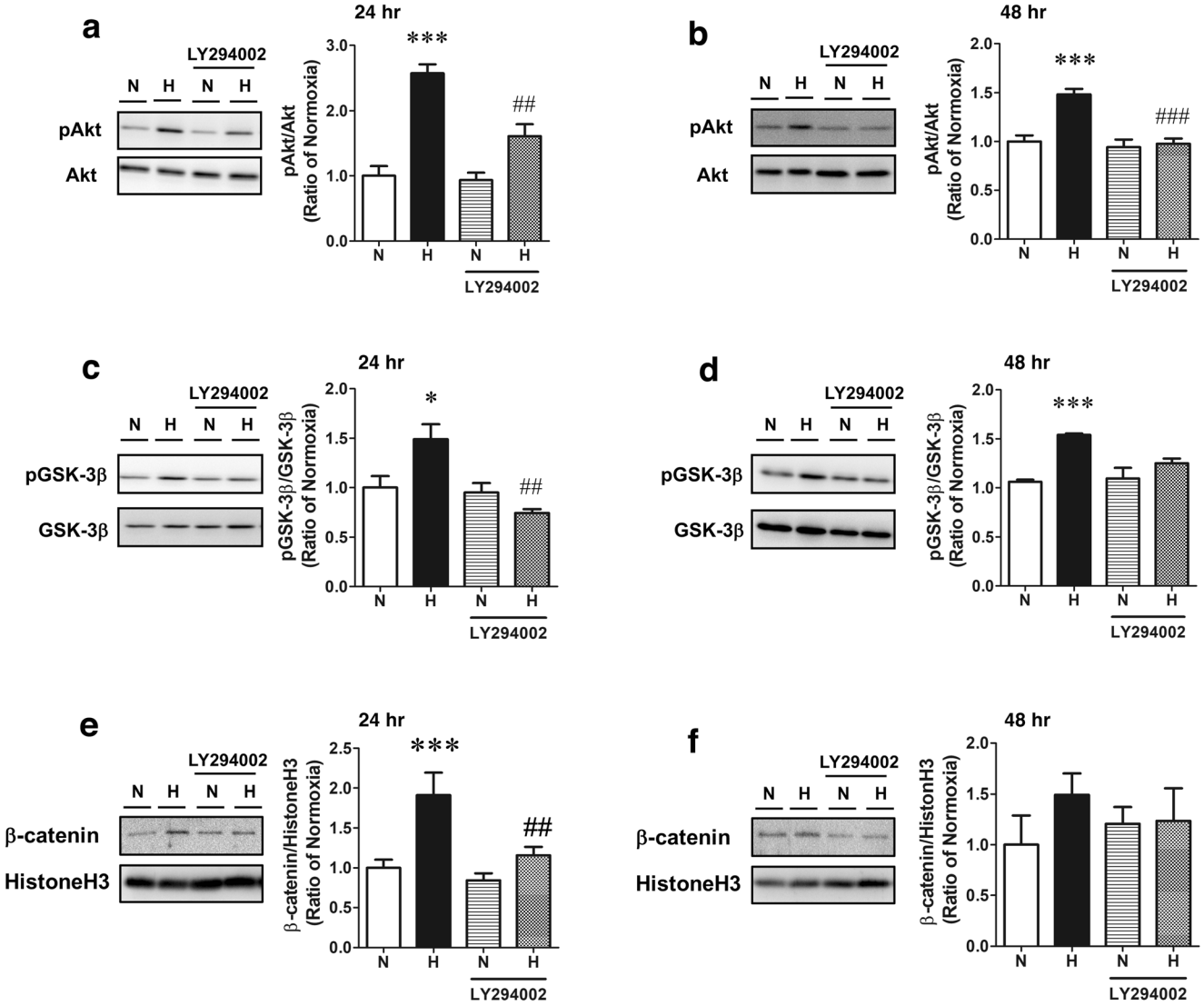

Fig. 4 Effect of LY294002 on Akt/GSK-3β signaling after hypoxic exposure. **a**–**f** Effects of LY294002 on levels of phosphorylated Akt (**a** and **b**), GSK-3β (**c** and **d**), and nuclear β-catenin (**e** and **f**) after 24 (**a**, **c**, and **e**) or 48 (**b**, **d**, and **f**) hours of normoxic (N) or hypoxic (H) exposure. Results are expressed as the mean ratio of the hypoxic group to the normoxic group ± SEM ($n = 4$ independent experiments). *Significant difference from the normoxic group ($P < 0.05$). ***Significant difference from the normoxic group ($P < 0.001$). ##Significant difference from the LY294002-untreated hypoxic group ($P < 0.01$). ###Significant difference from the LY294002-untreated hypoxic group ($P < 0.001$)

LY294002 (Fig. 5e, Bonferroni's post hoc test, $P > 0.05$ H versus H + LY294002 and $P < 0.05$ N + LY294002 versus H + LY294002). The expression of GFAP mRNA was not increased by hypoxic exposure compared with that under the normoxic condition (Fig. 5f). Also, there was no change in the expression of GFAP mRNA regardless of treatment or not with LY294002 (Fig. 5f).

Discussion

In previous studies, we demonstrated that cerebral ischemia increased the number of proliferating cells in the dentate gyrus of the hippocampus and that some of these cells expressed DCX, a marker of neural progenitors, and NeuroD1, which plays an essential role in neural differentiation [1, 5]. However, it has been reported that dysfunction of spatial learning and post-stroke depression-like symptoms are observed until day 28 after cerebral ischemia even though endogenous neurogenesis is enhanced on day 7 after it [14]. We assumed that if the activities of neural stem cells, including their proliferation and differentiation, could be further enhanced or sustained by some means, a new therapeutic target for treatment after cerebral ischemia might be identified. Thus, a better understanding of the profiles of neural stem cells under pathophysiologic

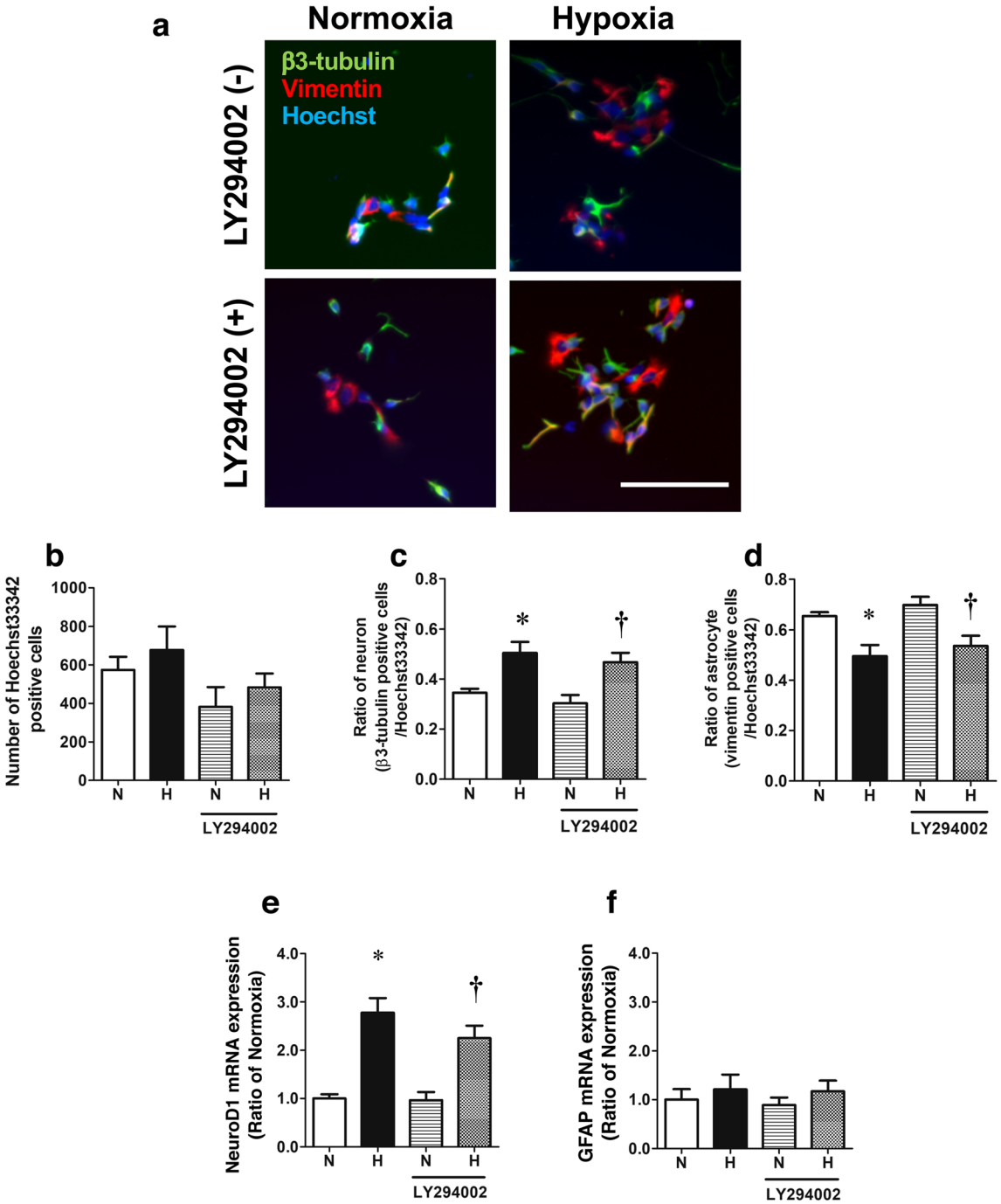

Fig. 5 Effects of LY294002 on the differentiation of neural stem/progenitor cells and mRNA expression of NeuroD1, which is a proneural basic helix-loop-helix transcription factor, in the differentiated cells. **a** Images obtained by staining for β3-tubulin (green), vimentin (red), and Hoechst33342 (blue) with (+) or without LY294002 (−) treatment. Scale bar, 100 μm. **b** Effects of normoxic (N) and hypoxic (H) exposure on the total number of Hoechst33342-stained cells with or without LY294002 treatment. **c** and **d** Effects of normoxic (N) and hypoxic (H) exposure on the number of β3-tubulin- (**c**) and vimentin-positive (**d**) cells with or without LY294002 treatment. Results are expressed as the mean ± SEM ($n = 4$ independent experiments). **e** and **f** Effects of normoxic (N) and hypoxic (H) exposure on the expression of NeuroD1 (**e**) and GFAP (**f**) mRNA in differentiated cells with or without LY294002 treatment. Results are expressed as the mean ratio of the hypoxic group to the normoxic group ± SEM ($n = 4$ independent experiments). *Significant difference from the normoxic group ($P < 0.05$). **Significant difference from the normoxic group ($P < 0.01$). †Significant difference from the normoxic group with LY294002 treatment ($P < 0.05$). ##Significant difference from the LY294002-untreated hypoxic group ($P < 0.01$)

conditions would be required. The objective of the present study was to determine the cellular mechanisms at play during the initial stage of neurogenesis processes, i.e., proliferation and neural differentiation, in neural stem cells

under the hypoxic condition, which mimics cerebral ischemia.

Neurogenesis is closely involved in some neurological diseases and is regulated by various factors [15–17]. Akt/GSK-3β signaling plays important roles in multicellular processes. For example, β-catenin is phosphorylated by GSK-3β and subsequently degraded by the ubiquitin-proteasome system. Non-phosphorylated β-catenin is stable in the cytoplasm and is translocated to the nucleus, where it acts as a key transcription factor of gene expression involving cell proliferation and differentiation. In the present study, the proliferation of neural stem/progenitor cells under the hypoxic condition was increased as compared with that under the normoxic one. Furthermore, the level of activated Akt, which is produced by phosphorylation of its Ser473, was increased after hypoxic exposure. Being downstream, GSK-3β is inactivated by being phosphorylated at its Ser9 by the activated Akt. Treatment with LY294002 inhibited the enhancement of cell proliferation under the hypoxic condition, due to inhibition of the phosphorylation of Akt at its Ser473 and GSK-3β at its Ser9. These results suggest that enhancement of proliferation of neural stem/progenitor cells under hypoxia involved Akt/GSK-3β signaling, at least in part.

We previously demonstrated that injection of exogenous neural stem cells improves the brain dysfunction after cerebral ischemia [8]. Noteworthy, these injected cells are observed around site of injury and are sustained in undifferentiated state. We suggested that injected neural stem cells might contribute to the improvement of brain dysfunction by secreting autocrine and/or paracrine factors rather than by replacing injured neuron. Moreover, neural stem cell-conditioned medium exerts a neuroprotective effect on cortical neurons after glutamate exposure [18]. Recruitment of neural stem cells may potentially be therapeutic after a stroke. In addition, it has been reported that therapeutic agents for neurological diseases, such as ethosuximide and lithium, enhance the proliferation of neural stem cells by activating Akt/GSK-3β signaling [19, 20]. Therefore, other agents that activate this signaling pathway may be of benefit for the treatment of cerebral ischemia.

We further examined the relationship between neural differentiation and Akt/GSK-3β signaling under the hypoxic condition. In accordance with other reports [21], neural differentiation was increased by hypoxic exposure in our study. Interestingly, there were no changes in neural differentiation regardless of treatment or not with LY294002 under hypoxia. These results imply that the enhancement of neural differentiation induced by hypoxic exposure was independent of the Akt/GSK-3β signaling pathway. However, our previous study indicated that the PI3K/Akt-dependent GSK-3β signaling pathway is involved in the expression of NeuroD1 on day7 after cerebral ischemia [5]. It is known that Wnt3a secreted by astrocytes enhances adult neurogenesis via NeuroD1 expression [22]. Also, Wnt3a cross-activates the PI3K/Akt signaling pathway and induces axon outgrowth and proliferation in NIH3T3 cells [23, 24]. Furthermore, neural differentiation is promoted by juxtacrin signaling between neural stem cells and astrocytes [25]. Taking such findings into account, we surmise that Akt/GSK-3β signaling, though not acting during the differentiation stage of cerebral ischemia-induced neurogenesis in vivo, may be involved in this differentiation effected by other types of cells, such as astrocytes, oligodendrocytes, microglia, and/or vasculature-related cells, in the central nervous system.

In conclusion, we demonstrated that hypoxia enhanced cell proliferation in neural stem/progenitor cells. This hypoxia-induced enhancement of proliferation involved at least the PI3K/Akt/GSK-3β signaling pathway, although hypoxia-induced cell proliferation was not completely inhibited by LY294002 treatment. However, neural differentiation under the hypoxic condition was not directly regulated through this signaling pathway. It might be supported by factors secreted from some other type(s) of cells and/or cell-cell interaction. Although enhancement of proliferation of endogenous neural stem cells through such signaling may be therapeutic to restore tissue damage and neurological dysfunction caused by cerebral ischemia, further studies will be needed to determine whether or not the differentiation of neural stem cells is affected by other cell types under the ischemic condition.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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