Full Paper

Endogenous Nitric Oxide Generation Linked to Ryanodine Receptors Activates Cyclic GMP / Protein Kinase G Pathway for Cell Proliferation of Neural Stem/Progenitor Cells Derived From Embryonic Hippocampus

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Abstract. Nitric oxide (NO) activates the cyclic GMP (cGMP) / protein kinase G (PKG) pathway during physiological processes in numerous types of cells. Here, we evaluated whether this NO/cGMP/PKG pathway is involved in the proliferation of neural stem/progenitor cells (NPCs) derived from the hippocampus of embryonic mice. In culture, the exposure to the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) significantly decreased the number of viable cells and 5-bromo-2′-deoxyuridine (BrdU) incorporation into the cells, as well as the levels of intracellular reactive oxygen species, extracellular NO_2, and intracellular cGMP. Like L-NAME, the soluble guanylate cyclase inhibitor $1H$-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and PKG inhibitor KT5823 also decreased cell viability and BrdU incorporation. The membrane-permeable cGMP analogue 8-bromo-cGMP partially abolished the L-NAME–induced decrease in the BrdU incorporation. BrdU incorporation was decreased by Ca^{2+}-channel blockers, including dantrolene, MK-801, ifenprodil, and nifedipine. Interestingly, the NO_2 level was decreased by dantrolene, but not by the other 3 blockers. L-NAME and ODQ attenuated phosphorylation of Akt, but not that of extracellular signal-regulated kinases or epidermal growth factor receptors. Our data suggest that endogenous NO generation linked to dantrolene-sensitive ryanodine receptors activates the cGMP/PKG signaling pathway for positive regulation of proliferation of hippocampal NPCs derived from embryonic mice.

Keywords: cyclic GMP / protein kinase G pathway, neural stem/progenitor cells, nitric oxide, proliferation, ryanodine receptor channel

Introduction

Reactive oxygen species (ROS) include the superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot HO$), nitric oxide (NO), and peroxynitrite ($ONOO^-$; Fig. 1). A large number of studies indicate that ROS-induced changes in the intracellular redox status influence cellular activities including signal transduction, metabolism, growth, and apoptosis, as well as cellular systems involved in detoxification. Particularly, NO has been implicated in various pathogenic conditions, such as inflammation, neurodegenerative disease, cardiovascular disease, and neoplasia. NO is produced by NO synthase (NOS), which exists as 3 different isoforms known as the neuronal isoform (NOS1, nNOS), inducible isoform (NOS2, iNOS), and endothelial isoform (NOS3, eNOS). NOS1 and NOS3 are activated by Ca^{2+} via interaction with calmodulin, whereas NOS2 is expressed in most types of infections caused by pathogens such as bacteria, protozoa, fungi, and viruses (1, 2). NO functions as an endogenous messenger in most cells. The major physiological target of NO is the cyclic GMP (cGMP)-synthesizing enzyme referred to as soluble guanylyl cyclase (sGC). Once sGC is activated by NO, the elevated level of intracellular cGMP activates cGMP-dependent protein
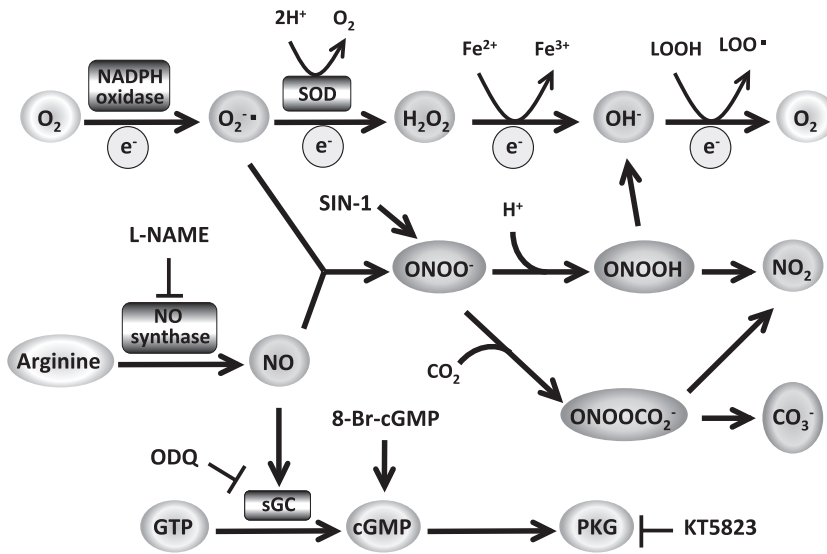
Fig. 1. ROS generation, NO/cGMP/PKG pathway, and their inhibitors/scavengers. ROS include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot HO$), nitric oxide ($\cdot NO$), and peroxynitrite ($ONOO^-$). L-NAME inhibits all subtypes of nitric oxide synthase (NOS). ODQ and KT5823 inhibit sGC and PKG, respectively. LOOH, fatty acids; LOO·, lipid peroxides; SOD, superoxide dismutase.

kinases (such as protein kinase G, PKG). Numerous studies suggest that phosphorylation cascades mediated by the NO/cGMP/PKG pathway are a widespread mechanism underlying NO-mediated physiological events in the nervous system (3 – 5). Moreover, NO reacts with the superoxide anion to form peroxynitrite, which has cytotoxic and pro-inflammatory effects in numerous cells (6). In addition to its role as a signal messenger, NO also modulates gene expression and causes post-translational modification of functional proteins through regulated expression of transcription factors and S-nitrosylation of the proteins, respectively (7, 8).

Accumulating evidence suggests that NO plays a critical role in neural development by affecting cell proliferation, migration, and differentiation (9, 10). In neuronal cell lines and embryonic cells derived from neural tissues, NO attenuates cell proliferation and facilitates cell differentiation (11). Further evidence for the anti-proliferative effect of NO on neural progenitor/stem cells (NPCs) comes from the observation that NOS inhibitors significantly enhance neurogenesis in the subventricular zone of adult mice and rats (11 – 13). Contrariwise, NO is known to be essential for proliferation in glial tumor cell lines (14). Further evidence for the essential role of endogenous NO and other ROS in the proliferation of NPCs comes from our recent findings that inhibitors of NOS and NADPH oxidase have the ability to attenuate proliferation of the NPCs derived from the hippocampus of embryonic mice (15). Based on these findings, we hypothesized that NO and peroxynitrite positively regulate the cell proliferation of NPCs. To date, however, there has been no evaluation of the mechanism underlying NO-mediated regulation of cell proliferation in the NPCs. In addition to this issue, no contradictory proposi-

tions for NO to act in survival and proliferation of different cells have been reported. To address these issues, we sought to elucidate the mechanisms underlying endogenous NO-mediated regulation of survival/proliferation in the NPCs derived from the hippocampus of embryonic mice.

In this study, we focused on the NO/cGMP/PKG pathway as the mechanism underlying the cell proliferation of the NPCs. To elucidate the involvement of the NO/cGMP/PKG pathway in cell proliferation, we examined the effects of inhibitors of NOS, sGC, and PKG on proliferation and its signals in the NPCs (Fig. 1). All inhibitors used had the ability to attenuate cell proliferation. To examine the involvement of Ca^{2+} signaling for activation of NOS in cell proliferation, we used blockers of Ca^{2+} channels, including L-type voltage-dependent Ca^{2+} channels (VDCC), NMDA-receptor channels, and ryanodine-receptor channels (RyR). All 4 blockers used had anti-proliferative effect on the NPCs; whereas the RyR blocker, but not the other 3 blockers, was capable of inhibiting endogenous NO generation in the NPCs. Taken together, our data show that the NO/cGMP/PKG signaling pathway linked to RyRs positively regulates cell proliferation of NPCs derived from the hippocampus of embryonic mice.

Materials and Methods

Materials

N^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-morpholinosydnonimine hydrochloride (SIN-1), 8-bromo-cGMP (8-Br-cGMP), MK-801, nifedipine, and dantrolene were purchased from Sigma-Aldrich Co.

(St. Louis, MO, USA). KT5823 and ifenprodil were purchased from Merck Chemicals (Darmstadt, Germany) and Wako Pure Chemical Industries, Ltd. (Osaka), respectively. 4-Hydroxynonenal was provided from Cayman Chemicals (Ann Arbor, MI, USA). 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) and Dulbecco's modified Eagle's medium : Nutrient Mixture F-12 (1:1, DMEM/F12) were supplied by Invitrogen Co. (Eugene, OR, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech (Rocky Hill, NJ, USA). Fetal cow serum (FCS) was from JRH Biosciences (Lenexa, KS, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and poly-L-lysine came from Nacalai Tesque, Inc. (Kyoto). The K-ASSAY Nitric Oxide Fluorometric assay and cGMP Complete EIA kit were obtained from Kamiya Biomedical Company (Seattle, WA, USA) and Assay Designs (Ann Arbor, MI, USA), respectively. Rabbit polyclonal antibodies against EGF receptor (EGFR) and phospho-EGFR (Tyr173, p-EGFR) were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); and rabbit polyclonal antibodies against Akt, phospho-Akt (Ser473, p-Akt), p44/42 mitogen-activated protein kinase (ERK), and phospho-ERK (Thr202/Tyr204, p-ERK) were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). HRP-conjugated anti-rabbit IgG antibody was purchased from DakoCytomation (Glostrup, Denmark). All other chemicals used were of the highest purity commercially available.

Cell culture

The present study was carried out in compliance with the Guideline for Animal Experimentation at Setsunan University with an effort to minimize the number of animals used and their suffering. Hippocampal NPC cultures were prepared from the hippocampus of 15-day-old embryonic mice as originally described by Yoneyama et al. (15) with several modifications (16). In brief, hippocampi were dissected from embryonic Std-ddY mice and then suspended in DMEM/F-12 supplemented with 10% (vol/vol) FCS by pipetting. After dissociation by treatment with 0.02% (wt/vol) EDTA for 10 min at room temperature, the cells were then washed twice with DMEM/F-12 supplemented with 10% (vol/vol) FCS by suspension and centrifugation at $500 \times g$ for 5 min and subsequently washed once again with DMEM/F-12 containing 0.6% (wt/vol) glucose, 15 mM sodium bicarbonate, 20 nM progesterone, 30 nM sodium selenite, 60 nM putrescine, and 100 μg/mL apo-transferrin. Finally, the cells were suspended in growth medium consisting of DMEM/F12-containing 0.6% (wt/vol) glucose, 15 mM sodium bicarbonate, 20 nM progesterone, 30 nM sodium selenite, 60

nM putrescine, 100 μg/mL apo-transferrin, 25 μg/mL insulin, 10 ng/mL EGF, and 10 ng/mL bFGF. These cells were seeded at a density of 6×10^4 cells/mL in 6-well dishes (Greiner bio-one, Germany) after counting viable cell numbers determined by the trypan blue exclusion test and were then cultured for a period up to 9 days in vitro (DIV) in the growth medium with a half medium change every 3 days as primary cultures of NPCs. The cells in the 9 DIV cultures were dispersed by using a NeuroCult Chemical Dissociation Kit (StemCell Technologies Inc., Vancouver, Canada), and then replated at a density of 6×10^4 cells/mL in 6- or 24-well dishes as secondary cultures. The cells were kept in the growth medium for various times up to 6 DIV under the same conditions as described for the primary cultures. Experiments in the present study were usually performed by using the secondary cultures unless otherwise indicated. The cultures were always maintained at 37°C in 95% (vol/vol) air / 5% (vol/vol) CO$_2$; and after seeding, the cells were exposed to no FCS at all to avoid possible influences of hitherto unidentified factors present in FCS.

Cultures of NPCs were prepared from the olfactory bulb and subventricular zone of adult Std-ddY mice (5 – 6 weeks of age) as described by Yoneyama et al. (17). Briefly, the olfactory bulb and subventricular zone of 10 mice were gently triturated, centrifuged at $500 \times g$ for 5 min, and enzymatically dissociated at 37°C for 30 min in 2 μg/mL papain, 0.5 mg/mL DNase, and 0.18 mg/mL neutral protease (Worthington Biochemical Co., Lakewood NJ, USA) in Dulbecco's phosphate-buffered saline containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 33 mM glucose. After gentle trituration and centrifugation at $500 \times g$ for 5 min, the cells were washed 2 times with DMEM/F-12 supplemented with 10% (vol/vol) FCS, 0.12% sodium bicarbonate, 100 U/mL penicillin, 0.1mg/mL streptomycin, 16.5 mM glucose, and 0.25 mM *N*-acetyl-L-cysteine. The cells finally obtained (30,000 cells/0.5 mL) were put into each well (1.9 cm^2) of a culture plate (Nalge Nunc dish, low cell bind, 24-well Cs7; Nunc, Denmark) and usually cultured for a period up to 9 or 12 DIV in DMEM/F12 containing 0.6% (wt/vol) glucose, 15 mM sodium bicarbonate, 20 nM progesterone, 30 nM sodium selenite, 60 nM putrescine, 100 μg/mL apo-transferrin, 25 μg/mL insulin, 20 ng/mL EGF, and 20 ng/mL bFGF as growth medium with a half medium change every 3 days. For neurosphere assays, the number of neurospheres (over 30 μm in diameter) was counted in each well of the culture plates.

ROS imaging

For determination of ROS, we used the fluorescent reagent CM-H$_2$DCFDA, which is sensitive to a variety of

ROS ($\cdot O_2^-$, H_2O_2, $\cdot HO$, NO, and $ONOO^-$). Cells were incubated with 5 μM CM-H$_2$DCFDA for 1 h in a 5% CO_2 / 95% air–humidified incubator. Immediately after the incubation, the cells were observed under a fluorescence microscope IX71 (Olympus, Osaka) equipped with a VB-7010 digital camera (Keyence, Osaka). Quantification of the mean signal intensity in individual cells was performed by using the software ImageJ.

Cell proliferation and viability

Cell proliferation was assessed in terms of 5-bromo-2′-deoxyuridine (BrdU) incorporation into cells during the culture period. Cells were exposed to 0.1 μM BrdU for 12 h and then centrifuged at $300 \times g$ for 10 min. After removal of the medium, the BrdU level in the cells remaining in the dish was determined by using a Cell Proliferation ELISA kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cell viability was determined by using the MTT assay. In brief, MTT solution (0.5 mg/mL in phosphate-buffered saline) was added to each well of the culture dishes; and then the cells were incubated for 2 h at 37°C. Subsequently, solubilizing solution (0.4 M HCl in isopropanol) equivalent to the MTT solution in volume was added, after which the absorbance at 570 nm was measured.

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) released into the medium (18). Cells were centrifuged at $600 \times g$ for 10 min, and then the culture medium was collected and stored at −80°C until the assay was performed. The LDH reaction was initiated by mixing 50 μL of the cell-free supernatant with a potassium phosphate buffer (pH 7.4) containing 0.1 mM NADH and 100 mM sodium pyruvate to a final volume of 252 μL in a cuvette. Immediately, the absorbance at 340 nm was measured at an interval of 30 s until 6 min after starting the reaction. LDH released into the medium was determined in triplicate.

Reverse transcription PCR (RT-PCR) analysis

Total RNA was isolated from the NPCs with Trizol by following the manufacturer's instructions (Invitrogen Co.). One microgram of total RNA was reverse-transcribed to prepare cDNA by using Oligo(dT)15 primer in accordance with the instructions for use of Ready-To-Go You-Prime First-Stranded Beads. An aliquot of cDNA was then amplified by using a 0.4 μM concentration of each primer set for the genes in 25 μL of reaction mixture containing a 0.2 mM concentration of each dNTP, 0.625 units *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, and 0.001% gelatin, with denaturation at 94°C for 1 min, elongation at 72°C for 1 min, and annealing under the conditions given in Table 1. The nucleotide sequence of the primers is also denoted

Table 1. Primers and conditions used for RT-PCR analysis

Gene	Upstream (5′-3′)	Downstream (5′-3′)	Product (b.p.)	Annealing temperature and time
NOS1	TCGAGAGCACCA	CTGGTGCTGGAAGTACCCAT	475	53.0°C 30 s
NOS2	GTGGTGACAAGCACATTTGG	GCAGAGTGAAAAGTCCAGCC	487	60.0°C 30 s
NOS3	CCAATGCAGTGAAGATCTCT	CATACCCCCACTTCTGTGCC	488	
PRKG1	AAAAATGAGCGAACTGGAG	GACCTCTCGGATTTAGTGAAC	273	55.0°C 30 s
PRKG2	GTTCGGAAGAGTGGAGCTTG	CTTTGTTAAGAATGACCTCGGG	488	
GUCY1A2	AAGGGTCAACCTGGACTCAC	ATAGATTCTCTTTCCTGCAGCC	538	50.0°C 30 s
GUCY1A3	AGAAAGACAAGCCGCAACAGAGT	GCAGCCGCTTTAATGATACCAG	485	
GUCY1B2	ACTGGACCAGTCTTAGCAGG	GCAGCCGCTTTAATGATACCAG	475	48.0°C 30 s
GUCY1B3	GAGAAGGGGCCATGAAGATTGTC	CCTCCGTGCCTGTATTTTTCCTG	419	50.0°C 30 s
RYR1	CTTGGTGGTGTAGACAGAGCAG	TCCTGGATGACGTACAAGTGTC	166	
RYR2	CCCCTACACTCTTCTGGATGAC	CTTCACCGCATCGGTCTTCTCT	208	44.7°C 60 s
RYR3	CGAACTCCTCAGCTACCTCTGT	GTGAGCGGAACTCTCTTGTCTT	188	
β-Actin	CCCAGAGCAAGAGAGGTATC	AGAGCATAGCCCTCGTAGAT	340	54.5°C 60 s

in Table 1. Reactions were carried out for a suitable number [25 – 30] of cycles with the use of a Thermal cycler. After the last cycle, a final extension step was done at 72°C for 10 min, and then the PCR products were analyzed by conducting 1% agarose gel electrophoresis.

NO$_2$ and cGMP levels

For measurement of the NO$_2$ level, the culture medium in cultures was separated from the cells by centrifugation at $610 \times g$ for 10 min. The NO$_2$ level was measured by using a K-ASSAY Nitric Oxide Fluorometric assay according to the manufacturer's instructions. The fluorescence intensity corresponding to the NO$_2$ level was measured with a fluoromicroplate reader (MTP-100F; Corona Electric Co., Ltd., Ibaraki) under excitation at 360 nm and emission at 450 nm.

For measurement of the cGMP level, cell lysates were prepared by treatment with 0.1 M HCl for 20 min. The cGMP level was measured by using a cGMP Complete EIA kit according to the manufacturer's instructions. The optical density corresponding to the cGMP level (at 405 nm) was measured with a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblot analysis

Cells were harvested and homogenized by sonication in 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, a 10 mM concentration of each protein phosphatase inhibitor (NaF and sodium β-glycerophosphate), and 1 mg/mL of each of the following protease inhibitors: (*p*-aminidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin, and antipain. The cell lysates were boiled at 100°C for 10 min in 10 mM Tris-HCl buffer (pH 6.8) containing 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.01% (wt/vol) bromophenol blue, and 5% (vol/vol) 2-mercaptoethanol, and then stored at −80°C until used. An aliquot (10 μg protein) of the lysates was loaded onto a 7.5% or 10% polyacrylamide gel for electrophoresis at a constant current of 15 mA/plate for 2 h at room temperature and subsequently blotted onto a polyvinylidene fluoride membrane previously treated with 100% methanol. After having been blocked with 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with primary antibodies against EGFR, p-EGFR, Akt, p-Akt, ERK, or p-ERK for 2 h at room temperature. After 3 washings with 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20 (5 min each time), the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Proteins reactive with the antibody were detected by using Western Lightning Chemolumi-

nescence Reagent Plus and then quantified with ImageQuant 400 (GE Healthcare, Buckinghamshire, UK). Protein concentrations were determined with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories).

Data analyses

All data were expressed as the mean ± S.E.M., and the statistical significance was determined by using the Bonferroni/Dunn or Dunnett's test.

Results

Endogenous NO/cGMP/PKG pathway

To determine the presence of NOS (NOS1, NOS2, and NOS3), sGC (GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3), and PKG (PRKG1 and PRKG2) in the cultures, we performed RT-PCR using the specific primers (Table 1). All subtypes of NOS were found in the cultures, with the highest level being that of NOS3 (Fig. 2a). In addition to NOS, subtypes of sGC and PKG were also found in the cultures. In the sGC subtypes, however, GUCY1A2 and GUCY1B2 were at a lower level than the other subtypes (Fig. 3a). Thus, all components for a functional NO/cGMP/PKG pathway were present in the NPCs cultured under these conditions.

To confirm the formation of endogenous NO in the cultures, we examined the effect of L-NAME on the level of intracellular ROS and extracellular NO$_2$ as a metabolic product of NO. Exposure of the cells to L-NAME at 5 mM for 24 h reduced the ROS-derived fluorescence intensity of CM-H$_2$DCFDA in the cells and NO$_2$ level in the medium to $46.0 \pm 6.5\%$ and $63.0 \pm 7.0\%$, respectively (Fig. 2: b and c). In addition to the formation of NO, we tested the NO-dependent production of cGMP in the cells. Expectedly, the intracellular cGMP level was markedly reduced to $18.0 \pm 14.5\%$ by exposure to L-NAME (Fig. 3b). These data showing that the inhibition of NOS by L-NAME dramatically blocked cGMP formation suggest that endogenous NO activated the NO/cGMP/PKG pathway in the NPCs.

NOS inhibitor, SIN-1, and 4-hydroxynonenal

To evaluate whether the endogenous NO affected the proliferation and survival of the NPCs, we used L-NAME at the concentration of 5 mM, which substantially inhibits all subtypes of NOS (19). Figure 4a shows phase-contrast images of neurospheres cultured for 2 and 4 DIV in the absence or presence of L-NAME. At 4 DIV, neurospheres cultured in the presence of L-NAME were smaller than those cultured without it. Quantitative analysis of viable cells by performing the MTT assay revealed that L-NAME at 5 mM significantly decreased the viability of cells cultured for 4 DIV (Fig. 4b). In addition to cell vi-
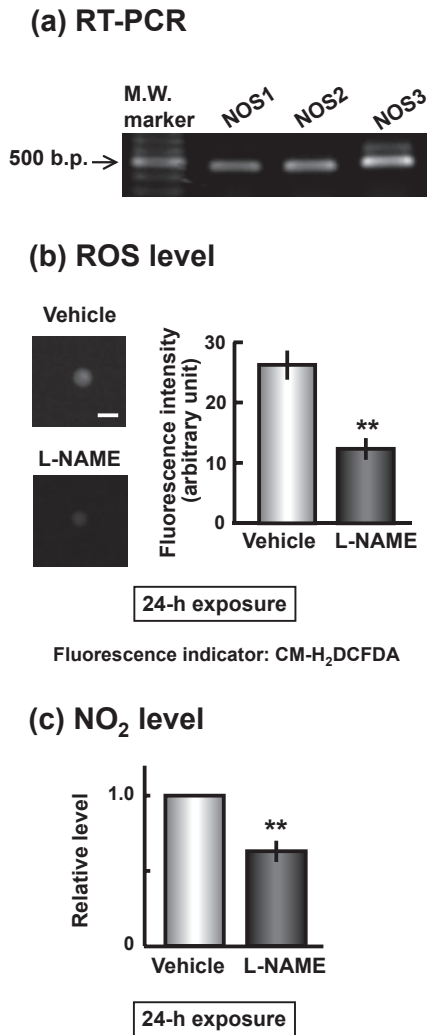
(a) RT-PCR

M.W. marker   NOS1   NOS2   NOS3

500 b.p. →

(b) ROS level

Vehicle

L-NAME

Fluorescence intensity (arbitrary unit)

30
20
10
0

Vehicle   L-NAME

**

24-h exposure

Fluorescence indicator: CM-H₂DCFDA

(c) NO₂ level

Relative level

1.0

0

Vehicle   L-NAME

**

24-h exposure

Fig. 2. NO production and its inhibition by L-NAME in the NPCs. Cells isolated from embryonic mouse hippocampus were cultured in the growth medium for 9 DIV and then dispersed in the growth medium. a) Total RNA was prepared from the cells and subjected to RT-PCR for determining NOS subtypes (NOS1, NOS2, and NOS3). These experiments were carried out at least 3 times with similar results under the same experimental conditions. b) The cells were exposed to either vehicle or 5 mM L-NAME in the growth medium for 24 h and then incubated with 5 μM CM-H$_2$DCFDA for 1 h for observation under a fluorescence microscope. The left panels denote typical fluorescence microscopic images of the cells. Scale bar = 10 μm. The right panel denotes quantitative data on the fluorescence intensity of the cells. Values are the means ± S.E.M. from 4 independent experiments. c) The cells were exposed to either vehicle or 5 mM L-NAME in the growth medium for 24 h. The culture medium was collected for measurement of the NO$_2$ level. Values are the means ± S.E.M. from 7 independent experiments. **$P < 0.01$, significantly different from the value obtained for the cells treated with vehicle alone.

(a) RT-PCR

M.W. marker   PRKG1   PRKG2   GUCY1A2   GUCY1A3   GUCY1B2   GUCY1B3   β-actin

500 b.p. →

(b) cGMP level

Relative level

1.0

0

Vehicle   L-NAME

**

24-h exposure

Fig. 3. cGMP production and its inhibition by L-NAME in the NPCs. Cells isolated from embryonic mouse hippocampus were cultured in the growth medium for 9 DIV and then dispersed in the growth medium. a) Total RNA was prepared from the cells and then subjected to RT-PCR for determining PKG subtypes (PRKG1 and PRKG2) and sGC subtypes (GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3). b) The cells were exposed to either vehicle or 5 mM L-NAME in the growth medium for 24 h, after which the cGMP level was measured. Values are the means ± S.E.M. from 4 independent experiments. **$P < 0.01$, significantly different from the value obtained for the cells treated with vehicle alone.

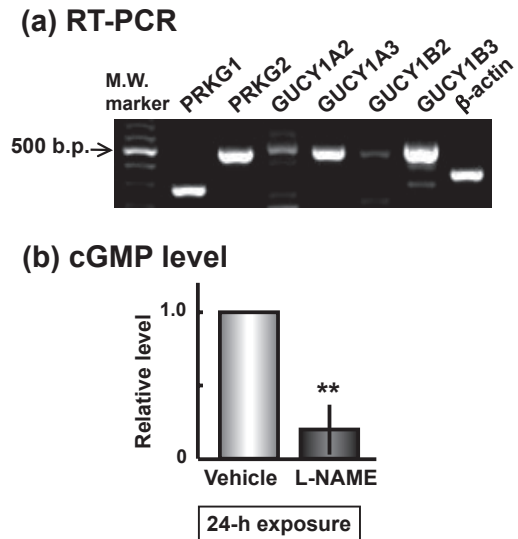ability testing, we conducted BrdU cell proliferation assay in the absence or presence of L-NAME. L-NAME attenuated BrdU incorporation in a concentration-dependent manner, significantly at 5 mM ($37.7 \pm 4.6\%$ vs. vehicle alone, $P < 0.01$, Fig. 4c), which was the level effective in the MTT assay. LDH released into the medium was measured after treatment with L-NAME for evaluating the possibility that the L-NAME–induced decrease in the cell viability and BrdU incorporation may have been due to cytotoxicity of L-NAME. Expectedly, L-NAME did not affect the extracellular LDH activity in the medium, suggesting that L-NAME was not cytotoxic toward the NPCs at least during the culture period examined (Fig. 4d).

SIN-1 is known to be a donor of both superoxide anion and NO, which transiently form peroxynitrite (20). A single exposure to SIN-1 at above 50 μM significantly increased BrdU incorporation into the cells cultured for 4 DIV (Fig. 5a). Furthermore, we tested the effect of 4-hydroxynonenal, a main product of lipid peroxidation by peroxynitrite, on the cell viability. At low concentrations of $0.01 - 1$ nM, 4-hydroxynonenal produced a significant increase in viable cells (Fig. 5b). Oppositely, 4-hydroxynonenal at higher concentrations of more than 100 μM was strongly cytotoxic, as described in previous reports (21).

(a)
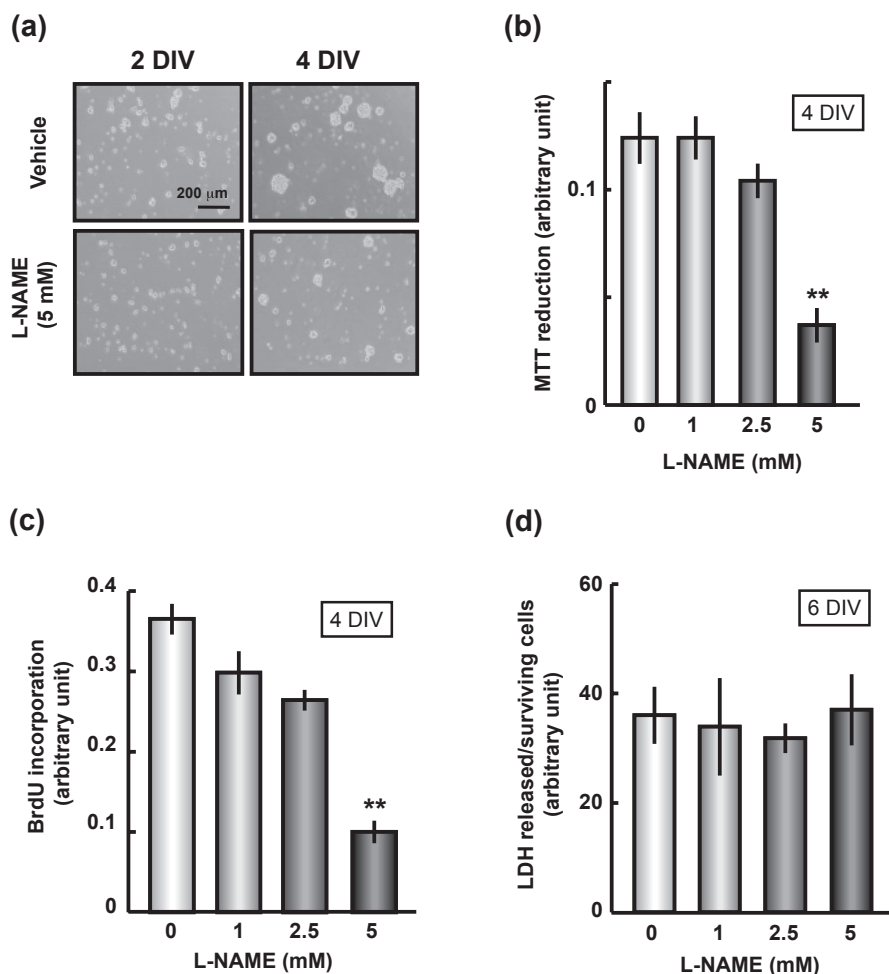

(b)

(c)

(d)

Fig. 4. Effect of L-NAME on proliferation and survival of the NPCs. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to either vehicle or L-NAME at different concentrations indicated in the growth medium for the various time periods indicated. a) Typical microscopic images of the cultures in the presence of vehicle alone or 5 mM L-NAME at 2 and 4 DIV. b) For determination of surviving cells, MTT the assay was carried out at 4 DIV. c) For determination of the proliferative activity of NPCs, BrdU incorporation into cells was measured by ELISA at 4 DIV. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. d) For determination of cell death, LDH activity in the culture medium was measured at 6 DIV. Values are the mean ± S.E.M. from 4 to 7 independent experiments. **$P < 0.01$, significantly different from each value obtained for the cells treated with vehicle alone (L-NAME = 0).

sGC and PKG inhibitors and cGMP analogue

To evaluate the involvement of sGC and PKG in the proliferation of the NPCs, we analyzed BrdU incorporation into the cells in the absence or presence of ODQ (sGC inhibitor) or KT5823 (PKG inhibitor) during the culture period. ODQ at 5 μM or above almost completely blocked the incorporation into cells at 4 DIV, whereas KT5823 attenuated the incorporation in a concentration-dependent manner at the concentrations of 0.5 μM and above (Fig. 6).

As 8-Br-cGMP is a membrane-permeating cGMP analogue (22), it activates PKG, which then causes the activation/inactivation of various cellular signals through phosphorylation of tyrosine and serine/threonine residues on their substrate proteins. In cultures at 4 DIV, the BrdU incorporation was significantly facilitated by exposure to 8-Br-cGMP alone (Fig. 7). In addition, 8-Br-cGMP at 1 mM had the ability to significantly but partially reverse the L-NAME–induced attenuation of the BrdU incorporation.

Ca²⁺-channel blockers

NOS1 and NOS3 are activated by increased intracellular Ca^{2+} and allow the release of NO from numerous types of cells. To elucidate which Ca^{2+} channels were involved in NO generation in NPCS and their proliferation, we examined the effect of blockers of various Ca^{2+} channels on the NO_2 level in the cells and BrdU incorporation into the cells. The Ca^{2+}-channel blockers tested included MK-801 (NMDA-receptor channel blocker), ifenprodil (NMDA-receptor NR2B antagonist), nifedipine (VDCC blocker), and dantrolene (RyR blocker, Fig. 8a). In addition to L-NAME, all 4 blockers used significantly attenuated the BrdU incorporation (Fig. 8b, left). Most interestingly, dantrolene, but not the other 3 blockers, significantly decreased the level of NO_2 (Fig. 8b, right). Evaluating the concentration-dependency of dantrolene with respect to the BrdU incorporation and NO_2 level, we found that dantrolene produced a concentration-dependent decrease in both, with a good correlation (Fig. 8c).
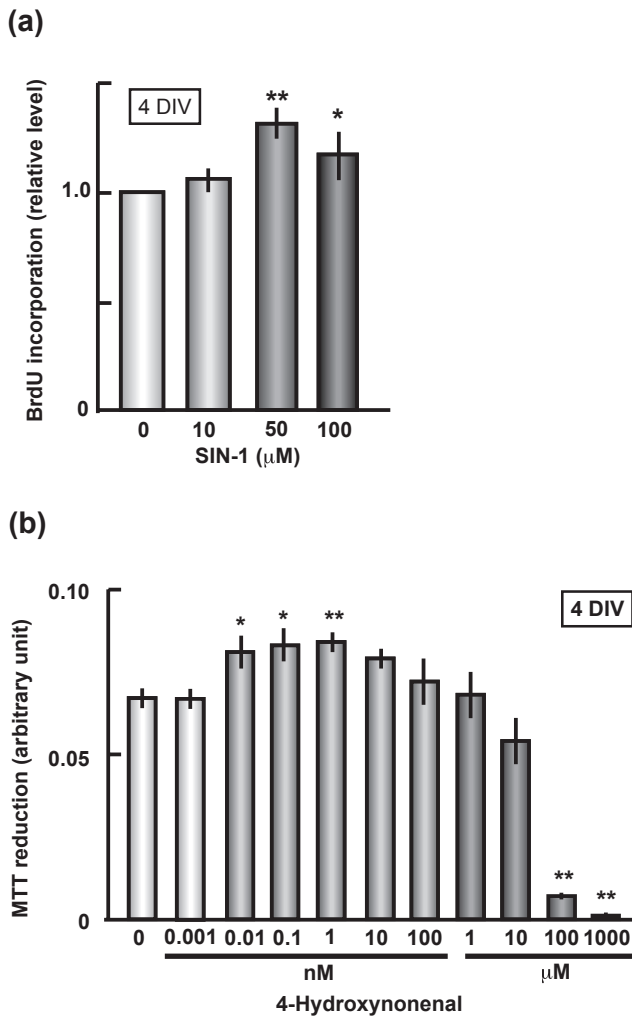
RyR is composed of at least 3 subtypes distributed

(a)

(b)

Fig. 5. Effect of SIN-1 and 4-hydroxynonenal on proliferation of the NPCs. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to vehicle, SIN-1, or 4-hydroxynonenal at the different concentrations indicated in the growth medium for 4 DIV. a) For determination of the proliferative activity of NPCs, BrdU incorporation into cells was measured by ELISA at 4 DIV in the absence or presence of SIN-1. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. b) For determination of surviving cells, the MTT assay was carried out at 4 DIV in the absence or presence of 4-hydroxynonenal. Values are the mean ± S.E.M. from 4 – 10 independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from each value obtained for the cells treated with vehicle alone (SIN-1 or 4-hydroxynonenal = 0).

throughout the skeletal muscle, cardiac muscle, and a variety of adapted muscles in other tissues. These include type 1 RyR (RyR1) expressed predominantly in skeletal muscle, type 2 RyR (RyR2) in cardiac muscle, and type 3 RyR (RyR3) in other muscular tissues and brain (23). We determined the subtypes expressed in the NPCs by

using RT-PCR analysis. RyR3 was the main receptor expressed in the cells cultured under normal conditions, though a slight expression of RyR2 was also observed in the cells (Fig. 8d). However, no RyR1 was detected under the same experimental conditions.

EGFR signaling

Because neurosphere formation by NPCs is strictly dependent on EGF, we evaluated whether L-NAME and ODQ would affect EGFR signaling at the early time window of the cultures. We initially investigated the effect of these inhibitors on EGFR tyrosine phosphorylation induced by EGF in the cells. Neither L-NAME nor ODQ had any effect on EGFR tyrosine phosphorylation following a 1-h treatment (Fig. 9). At the same time window, the phosphorylation state of Akt and ERK1/2 were analyzed to evaluate the activity of the phosphatidylinositol-3 kinase and mitogen-activated protein kinase as downstream effectors of EGFR. The phosphorylation state of Akt was significantly reduced by a 1-h treatment with L-NAME (Fig. 9: a and b) or with ODQ (Fig. 9: c and d). However, the phosphorylation state of ERK1/2 was not affected by either inhibitor. No significant change was observed in the level of EGFR, Akt, or ERK1/2 at the same time window.

NPCs derived from the subventricular zone and olfactory bulb of adult mice

To evaluate the effect of L-NAME on adult neurogenesis in the mouse brain, we prepared cultures of NPCs derived from the subventricular zone and olfactory bulb of adult mice. In the subventricular zone NPCs, BrdU incorporation by the cells was dramatically reduced by culturing them in the presence of L-NAME at 5 mM (Fig. 10a). Interestingly, the cells prepared from the olfactory bulb were insensitive to L-NAME at the same concentration (Fig. 10b).

Discussion

The essential finding of the present study is that RyR-mediated generation of NO was essential for proliferative activity through activation of the NO/cGMP/PKG signaling pathway in the NPCs derived from the hippocampus of embryonic mice. Although this signaling pathway has been well known as a common pathway for NO signals, we demonstrated for the first time that NO generation and its signaling pathway were mediated by RyR sensitive to dantrolene in the NPCs. Further evidence for the involvement of Akt in EGF signaling in the NO/cGMP/PKG signaling pathway–mediated regulation of the proliferation came from the findings that inhibitors of NOS and sGC down-regulated Akt phosphorylation in
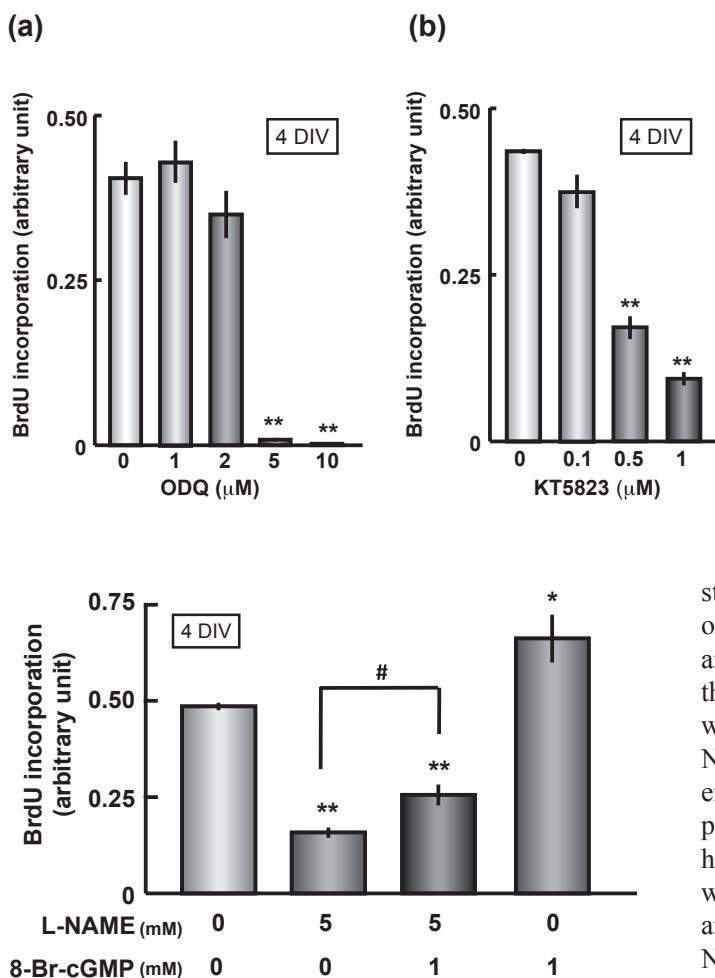
(a)   **(b)**

Fig. 6. Effect of ODQ and KT5823 on proliferation of the NPCs. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to vehicle, ODQ (a), or KT5823 (b) at the different concentrations indicated for 4 DIV. For determination of the proliferative activity of NPCs, BrdU incorporation into cells was measured by ELISA at 4 DIV. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. Values are the mean ± S.E.M. from 4 independent experiments. **$P < 0.01$, significantly different from each value obtained for cells treated with vehicle alone (drugs = 0).

Fig. 7. Effect of 8-Br-cGMP on L-NAME–induced attenuation of the cell proliferation. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to vehicle, L-NAME (5 mM), or 8-Br-cGMP (1 mM) in the growth medium for 4 DIV. For determination of the proliferative activity of NPCs, BrdU incorporation into cells was measured by ELISA at 4 DIV. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. Values are the mean ± S.E.M. from 4 independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from each value obtained for cells treated with vehicle alone (L-NAME/8-Br-cGMP = 0). #$P < 0.05$, significantly different from the value obtained for the cells treated with L-NAME alone.

the cells cultured under the normal conditions (Fig. 11).

Endogenous generation of ROS/NO

Numerous reports indicated that ROS/NO production is associated with most neurodegenerative diseases and brain ischemic insults (24) through intense activation of the apoptosis pathways in neuronal cells (25). Besides their well-known toxic effects, ROS/NO could be responsible for the modulation of various cellular functions under normal conditions (26). Recent reports demon-

strated that ROS play an important role in proliferation of proliferative cells (27, 28). Using ROS scavengers and an NADPH oxidase inhibitor, our previous study showed that proliferative activity is positively regulated by ROS, which are endogenously generated by activation of NADPH oxidase throughout the cell proliferation of embryonic hippocampal NPCs (15). In addition to the previous study, the current data show that the NOS inhibitor (L-NAME) attenuated the cell proliferation as well as caused a decrease in ROS-sensitive fluorescence and in the level of NO_2, which is a metabolic product of NO endogenously generated in the cultures.

How is NO generated in the NPCs? RT-PCR analysis in the present study evidently indicated the existence of 3 subtypes of NOS in the NPCs cultured under the conditions used. In general, NO is known to be generated by Ca^{2+}/calmodulin-dependent activation of NOS1 and NOS2 and enhanced expression of NOS2. Intracellular Ca^{2+} is increased by release from Ca^{2+} stores and influx via Ca^{2+} channels such as NMDA-receptor channels and VDCC. In the present study, we evaluated the effect of blockers of these Ca^{2+} channels on NO generation in the cultured NPCs. Most intriguingly, the RyR blocker dantrolene, but not the other blockers, had the ability to attenuate the generation of NO. Our current findings suggest that in the NPCs, NO was generated by activation of dantrolene-sensitive RyR, but not by that of VDCC and NMDA-receptor channels. Of the 3 types of RyR, abundant expression of RyR2 and RyR3 was observed in the NPCs in the present study. Because dantrolene is known to block RyR1 and RyR3 (29), it is possible that RyR3 contributed to NOS activation for NO generation in the NPCs. In mature cardiac muscle cells and smooth muscle cells, VDCC-mediated Ca^{2+} influx is well known to produce Ca^{2+} release from intracellular Ca^{2+} stores (Ca^{2+}-induced Ca^{2+} release) through coupling
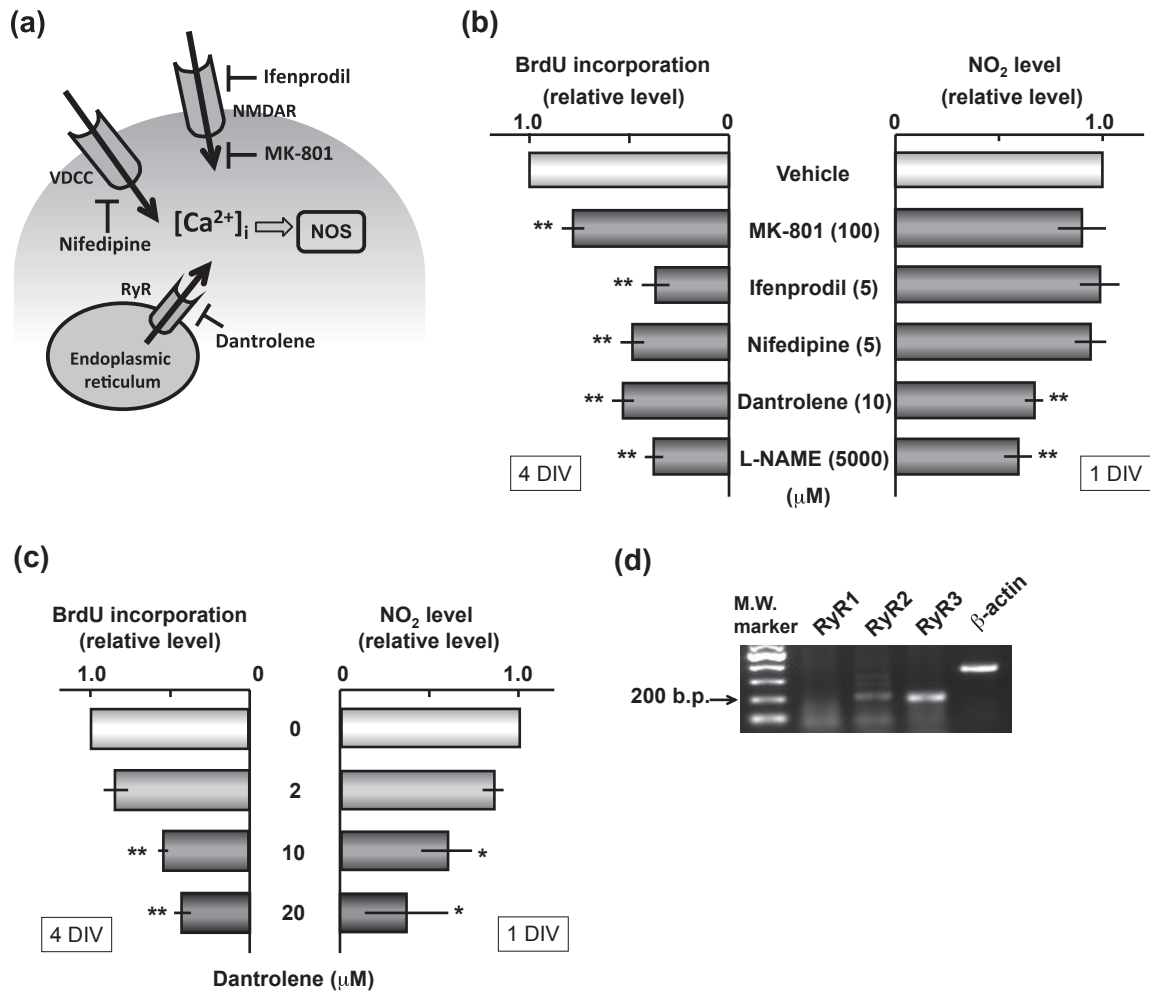
Fig. 8. Effect of Ca^{2+}-channel blockers on BrdU incorporation and NO_2 level in the NPCs. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to vehicle, L-NAME (5 mM), or Ca^{2+}-channel blockers in growth medium at the indicated concentrations. After a 24-h culture period, the culture medium was collected for measurement of its NO_2 level. After the cells had been cultured for 4 DIV, BrdU incorporation into them was measured by ELISA for determination of the proliferative activity of the NPCs. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. a) Functional roles of Ca^{2+}-channel blockers used are denoted as a schema. b) Effect of different Ca^{2+}-channel blockers. c) Effect of different concentrations of dantrolene. Values are the mean ± S.E.M. from 4 – 10 independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from each value obtained for cells treated with vehicle alone (drugs = 0). d) Total RNA was subjected to RT-PCR for determining ryanodine-receptor subtypes (RyR1, RyR2, and RyR3). These experiments were carried out at least 3 times, with similar results under the same experimental conditions.

between VDCC and RyR (30). Therefore, the present finding that the blockage of VDCC by nifedipine had no effect on the NO generation may support the idea that RyR3 in the NPCs was activated by a different mechanism from that operating in cardiac muscle cells. Indeed, there is a report that Ca^{2+} entering via VDCC fails to couple with Ca^{2+}-induced Ca^{2+} release in PC12 cells (31). Although Ca^{2+}-induced Ca^{2+} release from the intracellular Ca^{2+} stores (sarcoplasmic reticulum) is activated via Ca^{2+} entry via VDCC in the normal adult ventricular myocardium, in embryonic-ventricular myocytes Ca^{2+} entry via

T-type VSCC plays a significant role in stimulation of Ca^{2+}-induced Ca^{2+} release (32). Since we made no evaluation of the mechanism underlying Ca^{2+}-induced Ca^{2+} release in the NPCs, further evaluation is needed to elucidate the mechanism underlying NO generation through activation of Ca^{2+}-induced Ca^{2+} release via dantrolene-sensitive RyR in the NPCs. Because RyR channels play critical roles in the diverse physiologic and pathophysiologic cell processes that are controlled by Ca^{2+} release from intracellular stores (33), these channels represent potentially important pharmacologic targets for modulat-

(a)

(b)
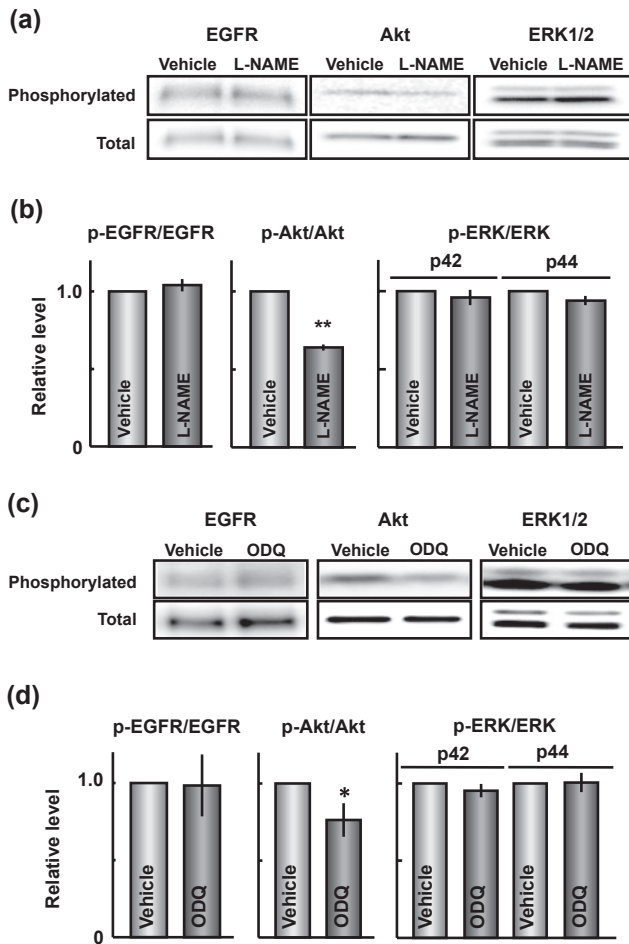

(c)

(d)

Fig. 9. Effect of L-NAME and ODQ on EGFR signaling in the NPCs. Cells were isolated from embryonic mouse hippocampus and then cultured in the growth medium for 9 DIV. After dispersal of the neurospheres, the cells were exposed to vehicle, 5 mM L-NAME (a, b) or 5 μM ODQ (c, d) in the growth medium for 1 h and then subjected to immunoblot analysis of EGFR, p-EGFR, Akt, p-Akt, ERK, and p-ERK. Values are the means ± S.E.M. from 6 independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from each value obtained for the cells treated with vehicle alone.

ing cell proliferation in the NPCs.

Ca²⁺ signaling

Calcium signaling mediates changes in gene expression, cell growth, development, survival, and cell death in numerous cells. As mentioned above, we demonstrated in the current study that proliferative activity in the NPCs was attenuated by Ca^{2+} blockers including MK-801, nifedipine, and dantrolene. These findings suggest that the proliferative activity is positively regulated by Ca^{2+} signaling mediated by NMDA-receptor channels, VDCC, and RyR. Of these Ca^{2+} channels, NMDA receptor channels–induced Ca^{2+} influx has been known to contribute to the proliferation in NPCs (34, 35). To data, however, there is no report about the down-stream signaling for NPC proliferation after Ca^{2+} influx mediated by NMDA-receptor channels, which has been well known to link NOSs for the generation of NO in neuronal cells (36). Interestingly, the current data suggest that NMDA-receptor channels contribute to the proliferation through other signaling pathways rather than NO signaling in the NPCs. In addition to NMDA-receptor channels, VDCC-mediated Ca^{2+} signaling for NPC proliferation also is unclear in the NPCs, although a recent report showed that VDCC has a critical role in NPC proliferation induced by hypoxia (37). Thus, Ca^{2+} signaling mediated by NMDA-receptor channels and VDCC must be further evaluated in detail.

NO/cGMP/PKG signaling pathway and peroxynitrite

In the present study, we showed for the first time that the NO/cGMP/PKG signaling pathway was involved in the proliferation of NPCs during development. Evidence for this involvement came from the findings that inhibition of NOS, sGC, or PKG by their respective inhibitor reduced the BrdU incorporation into the NPCs. In addition, L-NAME was capable of decreasing the level of
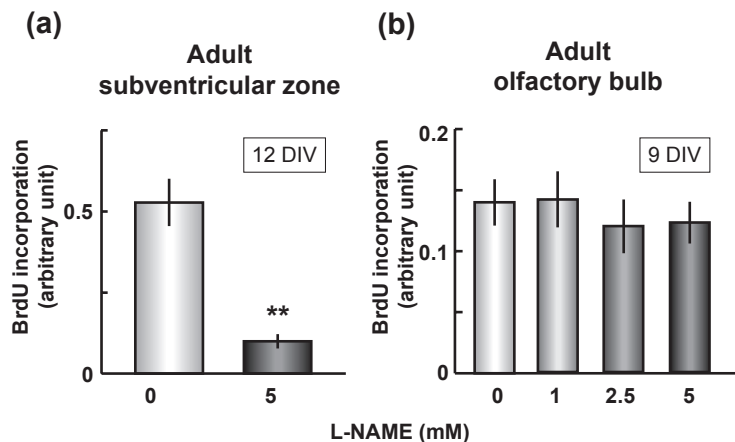
(a)

(b)

Fig. 10. Effect of L-NAME on proliferation of NPCs derived from the subventricular zone (a) and olfactory bulb (b) of mice. Cells were isolated from the subventricular zone and olfactory bulb of adult mice and then cultured separately in the growth medium in the presence of vehicle or 5 mM L-NAME at the different concentrations indicated for 12 and 9 DIV, respectively. For determination of the proliferative activity of NPCs, BrdU incorporation into cells was measured by ELISA at 4 DIV. The cells were treated with 10 μM BrdU in the growth medium for 12 h before harvesting the cells. Values are the mean ± S.E.M. from 4 independent experiments. **$P < 0.01$, significantly different from each value obtained for the cells treated with vehicle alone (L-NAME = 0).
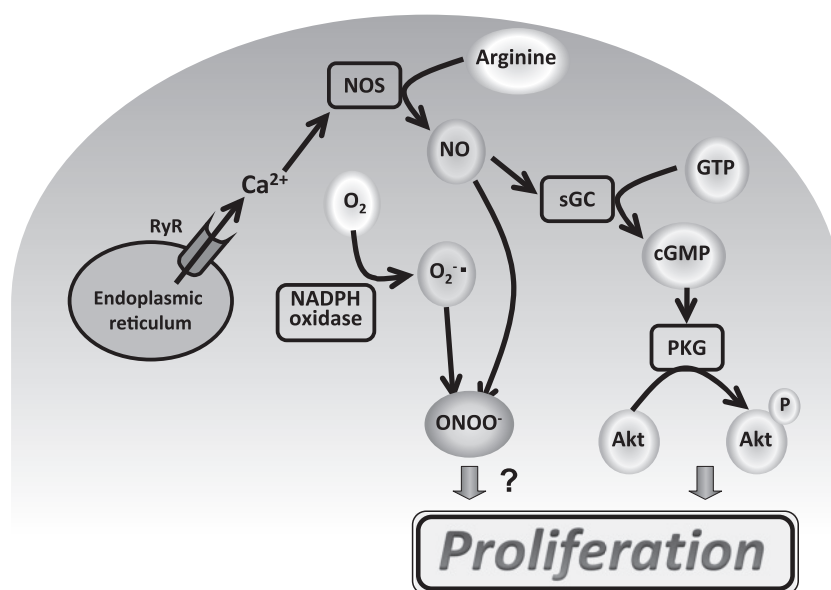
Fig. 11. Mechanism underlying NO-mediated proliferation of the NPCs derived from embryonic mouse hippocampus. In the NPCs, NOS is activated by Ca^{2+} released via activation of RyR in the endoplasmic reticulum. Once NO is generated, the intracellular level of cGMP is increased by the NO-activated sGC; and the former then activates PKG, which phosphorylates Akt, but neither EGFR nor ERK, as the proliferation signal in the NPCs. In addition to the NO/cGMP/PKG pathway, peroxynitrite could be, at least in part, involved in the proliferation of the NPCs.

cGMP. Hence, our data strongly support the proposition that a low concentration of endogenous NO activates sGC and elevates the intracellular level of cGMP. Indeed, NO at a low concentration is known to activate only cGC as its main target enzyme in neural cells (38). A second line of evidence was derived from a gain-of-function after exogenous application of cGMP. The membrane-permeable analog of cGMP enhanced the cell proliferation under the normal condition. In addition, this analog significantly and partially rescued L-NAME–induced attenuation of the cell proliferation. Our combined results imply that endogenous NO facilitated the cell proliferation through the production of cGMP, which then activated PKG, in the NPCs.

A possible different pathway for regulation of cell proliferation may be proposed, as the current data showed that 8-Br-cGMP was effective in partially reversing L-NAME–induced attenuation of the cell proliferation. As endogenous NO produces peroxynitrite through its reaction with superoxide anion in numerous types of cells, the finding that the peroxynitrite generator SIN-1 enhanced the cell proliferation suggests that peroxynitrite is a second candidate of NO signaling for positive regulation of the cell proliferation. There are a large number of reports that peroxynitrite has strong oxidizing properties, which are nitration of tyrosine and cysteine residues on many functional proteins as well as lipid peroxidation (39, 40). Indeed, our current data indicated that the main product of lipid peroxidation, 4-hydroxynonenal, at low concentration (0.01 – 1 nM) had the ability to enhance the cell proliferation, although the high concentration (more than 100 μM) was cytotoxic. Although many

proteins nitrated by peroxynitrite have been identified and evaluated for their functional significance under physiological and pathological conditions (41), the functional significance of nitrated and 4-hyroxynonenal–adducted proteins in proliferation and differentiation of NPCs is still unknown. In the future, further evaluation to identify nitrated proteins in the NPCs cultured in the present study may provide a hint for elucidation of the mechanism underlying NO/peroxynitrite-induced enhancement of proliferation in the NPCs.

EGFR signaling and NO/cGMP/PKG signaling

EGFR signaling controls cell migration, adhesion, apoptosis, cell-cycle progression, growth, and angiogenesis through activation of Akt, ERK1/2, and other molecules (42, 43). Particularly, Akt is a serine/threonine protein kinase and a downstream effector of phosphatidylinositol-3 kinase, both of which are part of a signaling pathway that can be initiated by EGFR activation (44). It has been shown that exposure to excess NO generated by donors produces a cGMP-independent decrease in cell proliferation of neuroblastoma cells through a reduction in EGFR phosphorylation (45). In the present study, conversely, we indicated that sufficient elimination of endogenous NO by L-NAME at the high concentration produced a cGMP-dependent decrease in cell proliferation through a significant decrease in Akt phosphorylation. However, L-NAME and ODQ had no effect on the phosphorylation of EGFR and ERK1/2. It thus is most likely that endogenous activation of NO/cGMP/PKG signaling produces predominantly Akt phosphorylation as a growth signal, without affecting EGFR activation, in

the NPCs under the normal condition. In other words, the integrity of the phosphatidylinositol-3 kinase / Akt pathway is necessary for neurosphere formation, which indicates that Akt is a major mediator in the proliferation and/or survival of neurosphere-forming cells derived from the embryonic hippocampus. Although there is no evidence for a direct or indirect linkage between the phosphatidylinositol-3 kinase / Akt pathway and PKG, a previous report indicating that excess NO promotes cell migration through PKG-dependent activation of the phosphatidylinositol-3 kinase / Akt pathway in endothelial cells (46) may support our proposition that NO-induced activation of PKG produces activation of the phosphatidylinositol-3 kinase / Akt pathway as proliferation/survival signaling in the NPCs

In summary and conclusion, we reported for the first time the NO/cGMP/PKG signaling–mediated enhancement of cell proliferation in the NPCs derived from the embryonic mouse hippocampus. Furthermore, endogenous NO generation was shown to be produced by activation of dantrolene-sensitive RyR. Hence, we conclude that activation of the NO/cGMP/PKG signaling by RyR plays a key role in the cell proliferation of the NPCs in the developing hippocampus. RyR-mediated signaling may be focused on as a new target for studies on neurogenesis and neurodevelopment in the future. To elucidate critical levels of Ca^{2+} for regulating the NPC proliferation, we must determine the change in the level of intracellular Ca^{2+} using highly-sensitive Ca^{2+} monitors in the future.

References

1   Nathan C. Inducible nitric oxide synthase: what difference does it make? J Clin Investig. 1997;100:2417–2423.

2   Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A. 2000;97:8841–8848.

3   Fiscus RR. Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. Neurosignals. 2002;11:175–190.

4   Akassoglou K. Nerve growth factor-independent neuronal survival: a role for NO donors. Mol Pharmacol. 2005;68:952–955.

5   Feil R, Hofmann F, Kleppisch T. Function of cGMP-dependent protein kinases in the nervous system. Rev Neurosci. 2005;16: 23–41.

6   Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996; 271:C1424–C1437.

7   Janssen YM, Matalon S, Mossman BT. Differential induction of c-fos, c-jun, and apoptosis in lung epithelial cells exposed to ROS or RNS. Am J Physiol. 1997;273:L789–L796.

8   Contestabile A. Regulation of transcription factors by nitric oxide in neurons and in neural-derived tumor cells. Prog Neurobiol. 2008;84:317–328.

9   Bicker G. STOP and GO with NO: nitric oxide as a regulator of cell motility in simple brains. Bioessays. 2005;27:495–505.

10 Bicker G. Pharmacological approaches to nitric oxide signalling during neural development of locusts and other model insects. Arch Insect Biochem Physiol. 2007;64:43–58.

11 Cheng A, Wang S, Cai J, Rao MS, Mattson MP. Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. Dev Biol. 2003;258:319–333.

12 Moreno-López B, Romero-Grimaldi C, Noval JA, Murillo-Carretero M, Matarredona ER, Estrada C. Nitric oxide is a physiological inhibitor of neurogenesis in the adult mouse subventricular zone and olfactory bulb. J Neurosci. 2004;24:85–95.

13 Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A, Estrada C. Nitric oxide decreases subventricular zone stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway. Stem Cells. 2007;25:88–97.

14 Tanriover N, Ulu MO, Isler C, Durak H, Oz B, Uzan M, et al. Neuronal nitric oxide synthase expression in glial tumors: correlation with malignancy and tumor proliferation. Neurol Res. 2008;30:940–944.

15 Yoneyama M, Kawada K, Gotoh Y, Shiba T, Ogita K. Endogenous reactive oxygen species are essential for proliferation of neural stem/progenitor cells. Neurochem Int. 2010;56:470–476.

16 Yoneyama M, Seko K, Kawada K, Sugiyama C, Ogita K. High susceptibility of cortical neural progenitor cells to trimethyltin toxicity: involvement of both caspases and calpain in cell death. Neurochem Int. 2009;55:257–264.

17 Yoneyama M, Kawada K, Ogita K. Enhanced neurogenesis in the olfactory bulb in adult mice after injury induced by acute treatment with trimethyltin. J Neurosci Res. 2010;88:1242–1251.

18 Shuto M, Seko K, Kuramoto N, Sugiyama C, Kawada K, Yoneyama M, et al. Activation of c-Jun N-terminal kinase cascades is involved in part of the neuronal degeneration induced by trimethyltin in cortical neurons of mice. J Pharmacol Sci. 2009; 109:60–70.

19 Stojanović R, Todorović Z, Vučković S, Neššić Z, Prostran M. N^{G}-nitro-L-arginine methyl ester potentiates the effect of aminophylline on the isolated rat hemidiaphragm. J Pharmacol Sci. 2003;92:157–162.

20 Darley-Usmar VM, Hogg N, O'Leary VJ, Wilson MT, Moncada S. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. Free Radic Res Commun. 1992;17:9–20.

21 Niki E. Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med. 2009;47:469–484.

22 Ishikawa T, Kaneko Y, Sugino F, Nakayama K. Two distinct effects of cGMP on cytosolic Ca^{2+} concentration of rat pancreatic beta-cells. J Pharmacol Sci. 2003;91:41–46.

23 McPherson PS, Campbell KP. The ryanodine receptor/Ca^{2+} release channel. J Biol Chem. 1993;268:13765–13768.

24 Love S. Oxidative stress in brain ischemia. Brain Pathol. 1999;9: 119–131.

25 Tan S, Wood M, Maher P. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem. 1998;71:95–105.

26 Remacle J, Raes M, Toussaint O, Renard P, Rao G. Low levels of reactive oxygen species as modulators of cell function. Mutat Research. 1995;316:103–122.

27 Buggisch M, Ateghang B, Ruhe C, Strobel C, Lange S, Wartenberg M, et al. Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase. J Cell Sci. 2007;120:885–894.

28 Schild L, Makarow P, Haroon F, Krautwald K, Keilhoff G. Distinct H_2O_2 concentration promotes proliferation of tumor cells after transient oxygen/glucose deprivation. Free Radic Res. 2008; 42:237–243.

29 Zhao F, Li P, Chen SR, Louis CF, Fruen BR. Dantrolene inhibition of ryanodine receptor Ca2+ release channels. Molecular mechanism and isoform selectivity. J Biol Chem. 2001;276: 13810–13816.

30 Kamishima T, Quayle JM. Ca^{2+}-induced Ca^{2+} release in cardiac and smooth muscle cells. Biochem Soc Trans. 2003;31:943–946.

31 Tully K, Treistman SN. Distinct intracellular calcium profiles following influx through N- versus L-type calcium channels: role of Ca^{2+}-induced Ca^{2+} release. J Neurophysiol. 2004;92:135–143.

32 Kitchens SA, Burch J, Creazzo TL. T-type Ca^{2+} current contribution to Ca^{2+}-induced Ca^{2+} release in developing myocardium. J Mol Cell Cardiol. 2003;35:515–523.

33 Berridge MJ, Bootman MD, Lipp P. Calcium-a life and death signal. Nature. 1998;395:645–648.

34 Kitayama T, Yoneyama M, Yoneda Y. Possible regulation by N-methyl-d-aspartate receptors of proliferative progenitor cells expressed in adult mouse hippocampal dentate gyrus. J Neurochem. 2003;84:767–780.

35 Joo JY, Kim BW, Lee JS, Park JY, Kim S, Yun YJ, et al. Activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells. J Cell Sci. 2007;120: 1358–1370.

36 Vincent SR. Nitric oxide neurons and neurotransmission. Prog Neurobiol. 2010;90:246–255.

37 Guo Z, Shi F, Zhang L, Zhang H, Yang J, Li B, et al. Critical role of L-type voltage-dependent Ca^{2+} channels in neural progenitor cell proliferation induced by hypoxia. Neurosci Lett. 2010;478: 156–160.

38 Garthwaite J. Concept of neural nitric oxide-mediated transmission. Eur J Neurosci. 2008;27:2783–2802.

39 Rubbo H, Trostchansky A, O'Donnell VB. Peroxynitrite-mediated lipid oxidation and nitration: mechanism and consequences. Arch Biochem Biophys. 2009;484:167–172.

40 Di Stasi AM, Mallozzi C, Macchia G, Petrucci TC, Minetti M. Peroxynitrite induces tyrosine nitration and modulates tyrosine phosphorylation of synaptic proteins. J Neurochem. 1999;73: 727–735.

41 Ischiropoulos H. Protein tyrosine nitration-an update. Arch Biochem Biophys. 2009;484:117–121.

42 Woodburn JR. The epidermal growth factor and its inhibition in cancer therapy. Pharmacol Ther. 1999;82:241–250.

43 Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther. 2004;3:465–472.

44 Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signaling. Exp Cell Res. 2003;284:31–53.

45 Murillo-Carretero M, Ruano MJ, Matarredona ER, Villalobo A, Estrada C. Antiproliferative effect of nitric oxide on epidermal growth factor-responsive human neuroblastoma cells. J Neurochem. 2002;83:119–131.

46 Kawasaki K, Smith RS Jr, Hsieh CM, Sun J, Chao J, Liao JK. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. Mol Cell Biol. 2003;23:5726–5737.