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

Masanori Yoneyama1,†, Koichi Kawada1,†,#, Tatsuo Shiba1, and Kiyokazu Ogita1,*

1Department of Pharmacology, Setsunan University Faculty of Pharmaceutical Sciences, Hirakata, Osaka 573-0101, Japan

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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 Nω-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 NO2, 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 Ca2+-channel blockers, including dantrolene, MK-801, ifenprodil, and nifedipine. Interestingly, the NO2 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 (·O2−), hydrogen peroxide (H2O2), hydroxyl radical (·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 Ca2+ 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

†These authors contributed equally to this work.
#Present address: Department of Pharmacology, Faculty of Pharmacy, Chiba Institute of Science, Shiomi-cho, Choshi, Chiba 288-0025, Japan
*Corresponding author. ogita@pharm.setsunan.ac.jp
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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.

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 Ca²⁺ signaling for activation of NOS in cell proliferation, we used blockers of Ca²⁺ channels, including L-type voltage-dependent Ca²⁺ 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*-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.
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-H2DCFDA) 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 Biomedica 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 \( \mu \)g/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 \( \mu \)g/mL apo-transferrin, 25 \( \mu \)g/mL insulin, 10 ng/mL EGF, and 10 ng/mL bFGF. These cells were seeded at a density of 6 \( \times \) 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 \( \times \) 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) CO2; 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 \( \mu \)g/mL papain, 0.5 mg/mL DNase, and 0.18 mg/mL neutral protease (Warthington 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, 0.1 mg/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 \( \mu \)g/mL apo-transferrin, 25 \( \mu \)g/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 \( \mu \)m in diameter) was counted in each well of the culture plates.

ROS imaging

For determination of ROS, we used the fluorescent reagent CM-H2DCFDA, which is sensitive to a variety of
ROS (\(\cdot O_2^-, H_2O_2, \cdot HO, NO, \) and ONOO\(^-\)). Cells were incubated with 5 \(\mu M\) 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 \(\mu M\) BrdU for 12 h and then centrifuged at 300 \(x\) 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 \(x\) g for 10 min, and then the culture medium was collected and stored at \(-80^\circ C\) until the assay was performed. The LDH reaction was initiated by mixing 50 \(\mu L\) 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 \(\mu L\) 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 \(\mu M\) concentration of each primer set for the genes in 25 \(\mu L\) 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream (5’-3’)</th>
<th>Downstream (5’-3’)</th>
<th>Product (b.p.)</th>
<th>Annealing temperature and time</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS1</td>
<td>TCGAGAGCAACCA</td>
<td>CTGGTGCTGGAAGTACCCAT</td>
<td>475</td>
<td>53.0°C 30 s</td>
</tr>
<tr>
<td>NOS2</td>
<td>GTGGTGACACGACACATTGG</td>
<td>GCAGAGTGAAAAGTCCAGCC</td>
<td>487</td>
<td>60.0°C 30 s</td>
</tr>
<tr>
<td>NOS3</td>
<td>CCAATGCAGTAAGATCTCTTCT</td>
<td>CATACCCCCCCTTTCTGC</td>
<td>488</td>
<td>55.0°C 30 s</td>
</tr>
<tr>
<td>PRKG1</td>
<td>AAAAATGAGCGAACCCTGGAG</td>
<td>GACCTCTCGGATTTAGTGAGAG</td>
<td>273</td>
<td>53.0°C 30 s</td>
</tr>
<tr>
<td>PRKG2</td>
<td>GTTCCGAAGAAGTGAGGCTTGT</td>
<td>CTTTGTATAGATGACCTGGG</td>
<td>488</td>
<td>50.0°C 30 s</td>
</tr>
<tr>
<td>GUCY1A3</td>
<td>AAGGATCAACCTGGACACTAC</td>
<td>ATAGATCCTTCTTTCTTCGCA</td>
<td>538</td>
<td>45.0°C 30 s</td>
</tr>
<tr>
<td>GUCY1B2</td>
<td>AGAAGAGGCAAGGCAACAGAGT</td>
<td>GCAGCCGTCCTTTATGACAG</td>
<td>485</td>
<td>48.0°C 30 s</td>
</tr>
<tr>
<td>GUCY1B3</td>
<td>ACTGGACCAGTCTTTCAGGAGG</td>
<td>GCAGCCGCTTTATGATACACG</td>
<td>475</td>
<td>50.0°C 30 s</td>
</tr>
<tr>
<td>GUCY1B3</td>
<td>GAGAAGGGGCCATGAAGAGTTGTC</td>
<td>CCTCCGTGCTCATTTTTCTG</td>
<td>419</td>
<td>50.0°C 30 s</td>
</tr>
<tr>
<td>RYR1</td>
<td>TCGGGTGGTGTAGACAGACAGCAG</td>
<td>TCCGGGATGACGTACAAGTGTAC</td>
<td>166</td>
<td>44.7°C 60 s</td>
</tr>
<tr>
<td>RYR2</td>
<td>CCCCCTACTCTTCTTGAGACGAC</td>
<td>CCTACGGCGATCGCTCTTCTCTCT</td>
<td>208</td>
<td>44.7°C 60 s</td>
</tr>
<tr>
<td>RYR3</td>
<td>CGAATCTCCAGTACCTCTTCT</td>
<td>GTGAGCGGAACTTCTTTCTT</td>
<td>188</td>
<td>54.5°C 60 s</td>
</tr>
</tbody>
</table>

\(\beta\)-Actin

CCCAGAGCAAGAGAGGTATC

AGAGCATAGCCCTCGTAGAT

340

45.5°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.

**NO2 and cGMP levels**

For measurement of the NO2 level, the culture medium in cultures was separated from the cells by centrifugation at 610 × g for 10 min. The NO2 level was measured by using a K-ASSAY Nitric Oxide Fluorometric assay according to the manufacturer’s instructions. The fluorescence intensity corresponding to the NO2 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-amidinophenyl)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 Chemoluminescence 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 NO2 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-H2DCFDA in the cells and NO2 level in the medium to 46.0 ± 6.5% and 63.0 ± 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 ± 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-
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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 ± 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 \( \mu M \) 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 \( \mu M \) was strongly cytotoxic, as described in previous reports (21).

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 \( \mu M \) CM-H2DCFDA for 1 h for observation under a fluorescence microscope. The left panels denote typical fluorescence microscopic images of the cells. Scale bar = 10 \( \mu m \). 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 NO2 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.
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 Ca2+ and allow the release of NO from numerous types of cells. To elucidate which Ca2+ channels were involved in NO generation in NPCs and their proliferation, we examined the effect of blockers of various Ca2+ channels on the NO2 level in the cells and BrdU incorporation into the cells. The Ca2+-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 NO2 (Fig. 8b, right). Evaluating the concentration-dependency of dantrolene with respect to the BrdU incorporation and NO2 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

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**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).
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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

![Graph](image.png)
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 demonstrated 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 NO2, 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 Ca2+/calmodulin-dependent activation of NOS1 and NOS2 and enhanced expression of NOS2. Intracellular Ca2+ is increased by release from Ca2+ stores and influx via Ca2+ channels such as NMDA-receptor channels and VDCC. In the present study, we evaluated the effect of blockers of these Ca2+ 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. In mature cardiac muscle cells and smooth muscle cells, VDCC-mediated Ca2+ influx is well known to produce Ca2+-induced Ca2+ release through coupling.

**Endogenous generation of ROS/NO**

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

![Graph](image)
Regulation of Proliferation by NO 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 Ca2+ entering via VDCC fails to couple with Ca2+-induced Ca2+ release in PC12 cells (31).

Although Ca2+-induced Ca2+ release from the intracellular Ca2+ stores (sarcoplasmic reticulum) is activated via Ca2+ entry via VDCC in the normal adult ventricular myocardium, in embryonic-ventricular myocytes Ca2+ entry via T-type VSCC plays a significant role in stimulation of Ca2+-induced Ca2+ release (32). Since we made no evaluation of the mechanism underlying Ca2+-induced Ca2+ release in the NPCs, further evaluation is needed to elucidate the mechanism underlying NO generation through activation of Ca2+-induced Ca2+ 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 Ca2+ release from intracellular stores (33), these channels represent potentially important pharmacologic targets for modulat-

**Fig. 8.** Effect of Ca2+-channel blockers on BrdU incorporation and NO2 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, 1-NAME (5 mM), or Ca2+-channel blockers in growth medium at the indicated concentrations. After a 24-h culture period, the culture medium was collected for measurement of its NO2 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 Ca2+-channel blockers used are denoted as a schema. b) Effect of different Ca2+-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.
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 date, 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 to NMDA-receptor channels, cGMP/PKG signaling pathway is known to contribute to the proliferation of NPCs. However, 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.

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**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.

**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).
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. Our combined results imply that endogenous NO facilitated the cell proliferation through the production of cGMP, which then activated PKG, in the NPCs.

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-hydroxynonenal–ad ducted 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

Regulation of Proliferation by NO


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