Neuregulin induces proliferation of neural progenitor cells via PLC/PKC pathway

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Abstract

Nestin-expressing neural progenitor cells (NPCs) have been isolated from hippocampus of brains and propagated with epidermal growth factor and basic fibroblast growth factor (bFGF). However, the underlying signaling mechanisms regulating NPC proliferation remain elusive. Here we showed that neuregulin\textsuperscript{b1} (NRG), like bFGF, effectively promoted the proliferation of hippocampus-derived NPCs and maintained the progenitor states of NPCs. Activation of protein kinase C (PKC), a downstream effector of phospholipase C (PLC), with 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA) mimicked the NRG-induced proliferation of NPCs. The synergic effect of TPA plus NRG on neurosphere growth further prompted us to find that NRG induced NPC propagation through PLC/PKC signaling pathway. ErbB4, an important functional receptor of NRG, had an interaction with PLC\textsubscript{c1} protein. In addition, inactivation of PLC pathway led to severe proliferative suppression of NPCs. Our study suggests that activation of PLC/PKC pathway plays an essential role in the NRG-induced proliferation of hippocampus-derived NPCs.

Keywords: Neural progenitor cell; Neuregulin; Proliferation; PLC; PKC; TPA

Nestin-expressing neural progenitor cells (NPCs) are defined as multipotential cells with a capacity for self-renewal. They are present in embryonic and newborn central nervous system (CNS) as well as in specific restricted regions of adult mammalian CNS, including the subventricular zone (SVZ) and the dentate gyrus of hippocampus [1]. NPCs in adult function to replace neural cells lost from normal turnover or due to damage [1]. In general, NPCs proliferate in response to epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) [2]. However, the underlying molecular mechanisms of NPC proliferation remain unclear.

Neuregulins (NRG) are members of EGF superfamily of growth and differentiation factors and transduce signals by activating ErbB receptor tyrosine kinases [3]. The NRG family includes more than 15 isoforms that differ at the C-terminal portion of their EGF-like domains. Transcripts encoding \(\beta\)-isoforms are enriched in neural tissue, whereas \(\alpha\)-isoforms predominate in mesenchyme [3,4]. Previous studies have demonstrated that NRGs are abundantly expressed during embryogenesis and down-regulated upon differentiation [5,6], and that the proliferation of neuroepithelia are damaged by selectively degrading NRG1 mRNA [7]. The exact function of NRG in NPC development, however, remains unclear.

The underlying signal transduction pathway that controls mitogen-induced proliferation of NPCs is still poorly understood. Previous studies have demonstrated that EGF or bFGF activate mitogen-activated protein kinase (MAPK) pathways in proliferation of NPCs [8,9]. In mammary epithelial cells, breast cancer cells, myelinating glial cells, or ovarian cancer cells, NRG has been reported to activate the phospholipase C (PLC), phosphotidylinositol-3-OH kinase (PI3K), and MAPK pathways in cell survival and proliferation [10–16]. In many types of cells, PLC is rapidly activated in response to growth factor stimulation and regulates cell proliferation and differentiation through generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3),
leading to protein kinase C (PKC) activation and intracellular calcium increase, respectively [17–19]. One specific aspect of PLC/PKC pathway relevant to our study is that PLC/PKC has been shown to play a vital role in regulating mouse embryonic stem-cell proliferation [20,21]. However, the relationship between activation of PLC pathway and proliferation of NPCs has not been clearly established.

Here we used a recombinant polypeptide containing the entire β-isofrom EGF-like domain of NRG1 (rHRGβ177–344) [22] to explore the role of NRG in regulation of NPC proliferation and the signaling mechanisms involved in this event. We found that NRG potently promoted NPC propagation by activating PLC/PKC pathway. Activation of PKC alone mimicked the NRG-induced proliferation, and inhibition of PLC pathway severely suppressed the growth of NPCs. We also observed that ErbB4, an important receptor of NRG, coimmunoprecipitated with PLCγ1 protein in proliferating NPCs. Our study suggests that PLC/PKC cascade plays a vital role in mitogen-induced NPC proliferation.

Materials and methods

Reagents and antibodies. We used a recombinant NRG (A gift from Dr. Mei Lin, University of Alabama) polypeptide containing the entire β-isofrom EGF-like domain of NRG-1 (rHRGβ177–344) [22]. The reagents were from Calbiochem (San Diego, CA; 12-O-tetradecanoylphorbol-13-acetate (TPA), 524400; Ly294002, 440202; Bis-indolylmaleimide I, BIS, 203291; and forskolin, 344271), Promega (Madison, WI; human recombinant bFGF, G5071; U0126, V1121), Peprotech (Rocky Hill, NJ; Neurotrophin-3 (NT-3), 450-03), Sigma (St. Louis, MO; poly- DD-lysine (PDL), P0899; 5-bromo-2′-deoxyuridine (BrdU), B5002), and Biomol International (San Diego, CA; U73122, ST391; U73343, ST392). Primary antibodies were from Lab Vision Corporation NeoMarkers (Fremont, CA; Ab-3, MS-304-PARX), Santa Cruz Biotechnology (Santa Cruz, CA; ErbB4, sc-283), Chemicon International (Temecula, CA; actin, MAB1501), Cell Signaling Technology (Beverly, MA; phospho-PLCα1, 2821; PLCγ1, 2822), BD biosciences PharMingen (San Diego, CA; Nestin, 556309), and Sigma (BrdU, B2531).

Neurosphere cultures. All procedures were approved by the Animal Experiment Committee of Chinese Academy of Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used. Neurospheres were cultured as described previously [23]. In brief, hippocampus was dissected from the brain of postnatal 0–24 h (P0) Sprague–Dawley rats and dissociated into single cells. The single cells were seeded in T25 flasks (Corning, NY) and grown in DMEM/F-12 (Invitrogen Corporation, MD) supplemented with N2 (containing 0.5 mg/ml insulin, 0.63 μg/ml progesterone, 1.611 mg/ml putrescine, 0.52 μg/ml sodium selenite, and 10 mg/ml human transferrin, all from Sigma). The cells were treated with or without NRG (0.1–10 nM), TPA (0.02–2 nM), bFGF (10 ng/ml), NT-3 (50 ng/ml) or forskolin (50 μM) for 6 days in vitro (DIV) to examine the formation of neurospheres. In some experiments, the cells were treated with NRG or bFGF supplemented with some reagents: Ab-3 (1 μg/ml), U73122 (4 μM), U73343 (4 μM), or BIS (5 μM).

Immunoprecipitation and immunoblotting. Proteins were extracted as described previously [24]. For immunoprecipitation, protein lysisates (~400 μg) were incubated directly with normal rabbit IgG or with the indicated antibodies overnight at 4 °C (the dilution of the antibodies was 1:100) on a rotating platform, and then incubated with protein A/G-agarose beads for 2 h at 4 °C on a rotating platform. After centrifugation, beads were washed 3–4 times with the RIPA buffer (containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM sodium orthovanadate, and protease inhibitors including 2 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin in phosphate-buffered saline (PBS)). Bound proteins were eluted and denaturalized with SDS sample buffer for 5 min at 95 °C and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was executed as described by Ma et al. [25]. For signaling pathway activity assay, neurospheres were pre-incubated with 4 μM U73122, 4 μM U73343, or 2 μM U0126 plus 5 μM Ly294002 (LY) for 15 min, respectively. Then the cells were treated with NRG or bFGF for 30 min before cell harvest. After protein extraction, equivalent amounts of protein were loaded in each experiment. Protein resolved on SDS-PAGE was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, MA). PVDF blots were blocked and then incubated with indicated primary antibodies. For immunoblotting, the dilution of the antibodies was 1:1000. After extensive washes, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Pierce, IL). Immunoreactive bands were visualized by SuperSignal West Pico enhanced chemiluminescent substrate (Pierce). All immunoblotting was repeated at least 2–3 times. For quantitative analysis, autoradiographic films were captured and analyzed with Molecular Analyst (Bio-Rad Laboratories, CA).

Immunocytochemistry. After 6 DIV, neurospheres were plated onto PDL (100 μM)-coated coverslips and allowed to adhere for 10 min. Cultured cells were fixed with 4% paraformaldehyde (in PBS) at room temperature for 20 min following extensive washes. Preparations were permeabilized with 0.1% Triton X-100 (in PBS) before staining, and incubated successively with 5% normal goat serum (NGS, in PBS), primary antibodies (in PBS with 3% NGS), and then fluorescein-conjugated secondary antibodies (in PBS with 3% NGS). For immunocytochemistry, the dilution of the primary antibodies was 1:100. Coverslips were mounted with PermaFluor Aqueous Mountant (Immunon Thermo Shandon, PA) containing 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI, D9542, Sigma), a nuclear marker. The number of DAPI positive cells represents the total cell number in each experiment. Preparations were imaged with a Leica DMRA microscope equipped with a cool snap camera Spot system (CTS, Chinetek Scientific, the Imaging Specialist).

Proliferation assay. Neurospheres were grown in the presence of 5-bromo-2′-deoxyuridine (BrdU, 10 μM) for 6 DIV and adhered on PDL-coated coverslips before cell fixation. Before cell permeabilization, neurospheres were immersed in 2 M HCl for 10 min and rinsed in 0.1 M borate buffer (pH 8.3) for 10 min. Cell proliferative rates were examined with BrdU antibody (1:500) according to immunocytochemistry procedure as described above.

TUNEL assay. TUNEL technology was used to reveal the apoptosis rates of floating neurospheres. Neurospheres were adhered for 10 min as above. TUNEL assay was performed following the manufacturer’s instructions after neurosphere adhesion. Cell death was quantified with the In Situ Cell Death Detection Kit (1,684 795, Fluorescein, Roche) according to manufacturer’s instructions. Finally, the cells were analyzed under a microscope. The apoptosis cells, which had cyan nucleus, were counted among ~2000 cells.

Statistical analysis. For immunocytochemistry statistical calculation, at least 20 nonoverlapping microscopic fields for each coverslip were analyzed and 2 coverslips for each experiment were selected. Each error bar represents mean ± SEM of data collected from at least three experiments. The statistical significance of differences between means was evaluated using Student’s t test and p values less than 0.01 were considered to be significant.
Results

To investigate the role of NRG in NPC proliferation, we cultured the dissociated P0 hippocampal NPCs [26] in NRG and demonstrated that NRG promoted proliferation of NPCs as a new mitogen. Activation of PKC, an important downstream effector of PLC, dramatically mimicked the NRG-mediated proliferation. The synergic effect of TPA plus NRG led to find that NRG-induced NPC propagation through PLC/PKC signaling pathway. In addition, PLC protein has an interaction with ErbB4 receptor. U73122, the specific antagonist of PLC, severely suppressed NPC propagation. These findings demonstrate that PLC/PKC pathway plays a vital role in NRG-induced NPC proliferation.

ErbB4 and Nestin are expressed in P0 hippocampus

To examine the role of NRG in hippocampal NPCs, we first analyzed the expression of ErbB4, an important functional receptor of NRG, in dissociated P0 hippocampal cells by immunolabel. ErbB4 proteins were mostly expressed in dissociated cells (Fig. 1A). Nestin protein, an intermediate filament typically present in neural progenitor cells, was expressed in 67.7 ± 1.7% of the dissociated cells (Fig. 1B). A lot of Nestin-positive (Nestin+) cells (red) co-labeled with ErbB4 (green) (arrow indicated). Scale bar represents 20 μm.

Fig. 1. Expression of Nestin and ErbB4 receptor in dissociated hippocampal cells. (A) ErbB4 (green) was abundantly expressed in a majority of dissociated hippocampal cells. (B) Nestin (red) was present in many dissociated cells. (C) DAPI (blue), a nuclear marker, indicated the total number of cells in one field. (D) The overlay of (A–C) showed that a lot of Nestin+ cells (red) co-labeled with ErbB4 (green) (arrow indicated). Scale bar represents 20 μm.

Fig. 2. NRG promoted the proliferation of NPCs. (A) NRG (0.1–10 nM) increased the size and number of neurospheres in a dose–response manner. The diameters of spheres from 20 fields and the total number of neurospheres in each field were collected for statistics. (B) The total number of neurospheres treated with NRG in each field was significantly increased, comparing with the untreated group (Con). NT-3 and bFGF were used as negative and positive controls, respectively. (C) Proliferation assay showed that NRG promoted more BrdU incorporation in NRG-treated neurospheres than in untreated neurospheres (Con). (D) NRG significantly reduced the proportion of TUNEL+ cells in neurospheres, as comparing with the untreated group (Con). bFGF was also used as a positive control in (C,D). Results are presented as means ± SEM and comparisons by Student’s t test are significant at p < 0.05 (indicated by *) or at p < 0.01 (indicated by **).
NRG promotes the proliferation and survival of NPCs

NRGs are highly expressed during embryogenesis, but down-regulated upon initiating differentiation [5,6]. Since there is a strong immunoreactivity of ErbB4 in Nestin-positive hippocampal cells, we speculate that NRG may have an important role in maintaining the progenitor state of NPCs.

To address this possibility, dissociated hippocampal cells were grown for 6 days in the presence of different concentrations of NRG. After a 6-day treatment, NRG of 0.1–10 nM increased the number and size of neurospheres in a dose-dependent manner (Fig. 2A). The neurospheres forming at 1 nM NRG, designated NRG-generated neurospheres, had larger size and more number of spheres (Fig. 2A, 62.8 ± 5.2 μm; 19 ± 3) than the untreated spheres (Fig. 2A, 38.2 ± 1.1 μm; 6 ± 2). Based on these results, we used 1 nM NRG in all of the following experiments if not specifically mentioned. To examine the biological effect of NRG, we also added soluble Ab-3, an antibody specifically binding to the NRG-recognizing site of ErbB4, to growing neurospheres for 6 days. This inhibitor of NRG bioactivity decreased mitosis of NPCs, resulting in a significant reduction in neurosphere number (Fig. 2B, 43.2% compared with untreated spheres (Fig. 2A, 60.6%)). In addition, Nestin and ErbB4 co-localized in a high proportion of cells in NRG-generated neurospheres (Fig. 3C). Interestingly, the proportion of Nestin+ cells significantly increased in dissociated hippocampal cells (not NRG-treated) (Fig. 3C, DHC, 67.7 ± 1.7%). In addition, Nestin and ErbB4 co-distributed in a lot of dissociated cells from NRG-generated neurospheres (Fig. 3D, arrow indicated). These results suggest that NRG not only promotes the proliferation of NPCs, but also maintains the expression of Nestin in the proliferating cells.

Activation of PKC is sufficient for proliferation of NPCs

During the study of NPC proliferation, we found that activation of PKC dramatically promoted the growth of neurospheres. A lately research reported that PLC/PKC was involved in embryonic stem-cell proliferation [21]. Less is known, however, about the role of PLC/PKC cascade in NPC proliferation.

Our results showed that TPA, a potent activator of classical PKC, intensively promoted the proliferation of NPCs (Fig. 4A, 64.5 ± 1.2 μm). For a 6-day treatment, the size and number of neurospheres were both increased in a dose-dependent manner with TPA of 0.02–2 nM (Figs. 4A and B). Introduction of another PKC activator, ryanodine, also promoted the growth of neurospheres, even though the proliferative effect of ryanodine was much weaker than that of TPA (data not shown). Bisindolylmaleimide I (BIS), a specific inhibitor of classical PKC, significantly blocked the TPA-induced growth of neurospheres (Fig. 4C). NT-3 (Fig. 2B) and forskolin (an agonist of adenylyl cyclase for increasing the levels of PKA/cAMP, Fig. 4C) did not obviously promote neurosphere formation. The proliferative effect
Fig. 4. TPA dramatically promoted the proliferation of NPCs. (A,B) TPA (0.02–2 nM) increased the size and number of neurospheres in a dose-dependent manner. (C) TPA potently initiated formation of neurospheres, while BIS (5 μM), a specific inhibitor of classic PKC, suppressed the TPA-induced growth of neurospheres. Forskolin (50 μM), an agonist of adenylate cyclase, did not initiate the formation of neurospheres. The effect of TPA (2 nM) and NRG (1 nM) did not synergize in high concentrations. (D) TPA (0.07 nM) and NRG (0.3 nM) in low concentrations had a synergic effect on proliferation, and the effect of 0.07 nM TPA plus 0.3 nM NRG was as potent as that of 1 nM NRG. *P < 0.01 (indicated by **).

of TPA indicated that PLC/PKC signaling pathway plays a vital role in regulating NPC proliferation.

To elucidate whether TPA and NRG go through the same pathway to regulate NPC proliferation, we supplied NPC with both TPA and NRG. When cultured cells were treated with TPA plus NRG in high concentrations, the proliferative effect of 2 nM TPA plus 1 nM NRG (Fig. 4C, TPA + NRG, 68.7 ± 1.8 μm) resembled the effect of 2 nM TPA (Fig. 4C, 64.5 ± 1.2 μm) or 1 nM NRG (Fig. 4C, 67.0 ± 1.7 μm) separately. However, in lower concentrations, the proliferative activity of 0.07 nM TPA and 0.3 nM NRG folded (Fig. 4D, 65.5 ± 1.3 μm), and the effect of 0.07 nM TPA plus 0.3 nM NRG was much more potent than that of 0.07 nM TPA (Fig. 4D, 50.5 ± 0.8 μm) or 0.3 nM NRG (Fig. 4D, 48.4 ± 0.9 μm) separately. The synergistic effect of TPA plus NRG in low concentrations was as powerful as that of NRG in high concentration (Fig. 4D, 1 nM NRG, 60.5 ± 1.1 μm). These results suggest that NRG and TPA promote proliferation through the same signaling pathway, and PLC/PKC cascade may be the promising signaling pathway involved in regulating NRG-induced NPC proliferation.

NRG activates PLC/PKC pathways in proliferating NPCs

Next we further investigate the role of PLC/PKC signaling pathway involved in the proliferative capacity of NRG. In many cell populations, NRG turns on the U73122-sensitive PLC/PKC [14] cascade. It is unclear whether and how PLC/PKC pathways are involved in the proliferative event of NPCs induced by NRG.

As shown in Fig. 5, specific activation of PLC pathway induced by NRG was measured with various inhibitors by immunoblotting with anti-phospho-PLCγ1 (p-PLCγ1) antibody. Cells were separately pre-incubated with different inhibitors for 15 min, and then the cells were treated with NRG for 30 min before protein extraction. Densitometric analysis revealed that at least threefold increase in PLCγ1 phosphorylation over basal level followed the NRG exposure. The capacity of NRG to activate PLCγ1 protein resembled that of bFGF, which was a well-established activator of PLC pathways in cell proliferation [30–33]. The specific PLC antagonist, U73122, potently inhibited the NRG-induced activation of PLCγ1, whereas U73343, the inactive
structural analog of U73122, did not obviously affect the activation of PLCγ1 (Fig. 5A). Pretreatment with U0126 and Ly294002 (LY), specific inhibitors of MAPK and PI3K, respectively, also did not significantly influence the NRG-induced phosphorylation of PLCγ1 protein. PLCγ1 blots were used as a loading control of phosphorylated PLCγ1 blots. bFGF was used as an activator of PLCγ1 protein. Positions of molecular weight markers were indicated on the left of the figures. (B) Coimmunoprecipitation data showed that rabbit polyclonal anti-ErbB4 and anti-PLCγ1 antibodies pulled down PLCγ1 and ErbB4, respectively. This result indicates that ErbB4 and PLCγ1 had an interaction in proliferating NPCs. Rabbit normal IgG (RN IgG) was used as a negative control for rabbit polyclonal anti-ErbB4 and anti-PLCγ1 antibodies.

We also used the protein lysate extracted from NRG-generated NPCs to examine the protein–protein interaction between PLCγ1 and ErbB4 proteins by co-immunoprecipitation assay. We used rabbit polyclonal anti-PLCγ1 and anti-ErbB4 antibodies to target ErbB4 and PLCγ1, respectively. The immunocomplex was precipitated as described in Materials and methods. Coimmunoprecipitation results showed that PLCγ1 and ErbB4 molecules interacted between each other in proliferating NPCs (Fig. 5B). Some of other protein bands may be the nonspecific binding products, since they also appeared in the control (rabbit IgG immunoprecipitates). These data indicated that NRG specifically promoted NPC proliferation through PLC/PKC signaling pathway.

**Inactivation of PLC severely suppresses the proliferation of NPCs**

As aforementioned, activation of PKC is sufficient for NPC proliferation. Then we further investigated whether PLC/PKC pathway was necessary for the NRG-induced proliferation. Cultured cells were treated with NRG plus U73122 or U73343 for 6 DIV, and the formed neurospheres were imaged and analyzed. We found that U73122 intensively inhibited the NRG-induced growth of neurospheres (Fig. 6A, 33.3/C6 1.5 µm), while U73343, the inactive analog of U73122, did not significantly affect the NRG-induced growth (Fig. 6A, 58.5/C6 1.3 µm), comparing with the growth induced by NRG alone (Fig. 6A, 58.6/C6 1.5 µm). Furthermore, the inhibitory effect of U73122 on the bFGF-induced proliferation resembled that of U73122 on the NRG-induced proliferation (Fig. 6B). We found that NRG also activated MAPK/Erk and PI3K/Akt pathways in proliferating NPCs by immunoblotting (data not shown), but the inhibitory effect of MAPK or PI3K inhibitors, such as U0126 or LY, on growth of neurospheres was much weaker than that of U73122 (data not shown). Our results suggest that PLC/PKC cascade plays an important role in mitogen-induced NPC proliferation.

**Discussion**

Our study demonstrated that NRG promoted the isolated Nestin-positive NPCs to form colonies as a new identified mitogen. In keeping with this, ErbB4 receptor...
was abundantly expressed in neurospheres formed from single NPCs. Previous study showed that soluble ErbB3 (which competes with endogenous ErbB3 receptor and blocks NRG signal transduction) decreased proliferation and increased apoptosis, indicating the essential role of NRG1 in striatum NPC proliferation [5]. However, GGF2, an isoform of NRGs which they used, had no significant effect on NPC proliferation [5]. As we know, ErbB3 has no intact function to transduce NRG signal if it does not form dimers with other NRG receptors [3], so we focus to study the effect of ErbB4, the full functional receptor of NRG [3]. Cellular interactions mediated by NRGs orchestrate critical development of several cell types and organs, including the heart, peripheral nervous system (PNS), and CNS [3,34–36]. In CNS, NRGs are important neuron-derived factors, which control many important neural activities, such as oligodendrocyte differentiation, neuromuscular synapse activity, neuron migration, and synaptic plasticity [3,34–36]. However, no evidence has shown the direct function of NRG in proliferation of NPCs, even though Calaoa et al. [5] have speculated the proliferative effect of NRG in NPCs. In this study, we used a recombinant NRGβ1 polypeptide containing only the active EGF-like domain to demonstrate directly the proliferative role of NRG in NPCs. Using this peptide also allowed us to avoid selecting the exact functional full-length molecule from a great diversity of NRG isoforms.

Like bFGF [37], NRG effectively induced proliferation of NPCs as a mitogen and an anti-apoptosis factor in our study, and PLC/PKC signal pathway was very essential for NPC proliferation. In response to various extracellular stimuli, including growth factors, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) producing DAG and IP3 to activate PKC and increase intracellular free calcium, respectively, and then implicates in many physiological functions [38]. Although PLC/PKC has been shown to play a vital role in regulating mouse embryonic stem-cell proliferation [21], no evidence has shown that activation of PLC/PKC directly regulates NPC proliferation. In our study, application of an agonist of PKC, TPA, was sufficient to induce the growth of neurospheres, while the specific antagonist U73122 of PLC, inhibiting cytosolic Ca2+ flux and PKC activation, potently suppresses NPC proliferation. However, activation of MAPK and PI3K by Insulin (data not shown) and NT3, respectively, could not initiate the formation of neurospheres. Hormonal stimulation of cAMP and the cAMP-dependent protein kinase PKA can positively or negatively regulate cell growth by multiple mechanisms [39]. PKA activation (after treatment with forskolin) in our assay did not modify cell proliferation even when combined with insulin and NT3 (data not shown). Pharmacological effect of U73122 further indicates that, in the growth of neurospheres, PLC/PKC pathway is more important than MAPK and PI3K pathways.

In our study, PLCγ1 has been proved to be coimmunoprecipitated with ErbB4. That one natural ErbB4 isoform mediating proliferation through non-PI3K pathway while another isoform mediating survival or chemotaxis though PI3K pathway indicate that alternative ErbB4 isoforms play various roles by coupling dissimilar downstream pathways [40]. Although we have not identified which isoform of ErbB4s interacts with PLCγ1 protein and regulates NPC proliferation in our study, we have demonstrated an important and direct physiological role of NRG in proliferation of hippocampus-derived NPCs through PLC/PKC cascade.

As we know, NT-3 and brain-derived neurotrophic factor (BDNF) and Trk receptors also activate PLC/PKC pathways [41–44], but they function to reduce neural progenitor cell proliferation and increase the differentiation of oligodendrocytic and neuronal lineages, respectively [45–48]. Cell proliferation in the developing CNS reflects a complicated interaction of positive (mitogenic growth factors, or activators of PKC) and negative (anti-proliferative factors, or agonists of PKA) regulators. As yet, it is still hard to exactly describe the mitogen-induced proliferative activity with the known signaling knowledge. Because many receptors regulated by different extrinsic stimuli share a common pool of downstream transduction molecules, an important strategy for ensuring specificity is to have further study in the multi-factor controlled NPC development.

In short, we concluded that NRG, acting via PLC/PKC cascade, is a new growth regulatory signal that controls proliferation of NPCs isolated from early postnatal hippocampus. Our study may have made some efforts to understand some profiles of the development of NPCs.

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