The generation of neurons is regulated by several diffusible signals including the fibroblast growth factor-2 (FGF-2), transcription factors such as NRG-1, and the brain-derived neurotrophic factor, which bind receptor-tyrosine kinases (1–3). FGF-2 is a potent mitogen present in the cerebral cortex throughout neurogenesis (3, 4) and in the neural precursor cells isolated from the embryonic telencephalon (5). Other signals caused by binding of an agonist for the muscarinic acetylcholine receptor, a G protein-coupled receptor (GPCR) (6), as well as gp130 ligands (7), have also been shown to stimulate DNA synthesis in neural precursor cells in the ventricular zone. Receptor-tyrosine kinases and GPCRs can both activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and there is evidence that FGF-2 utilizes a G protein to transmit signals to the MAPK pathway (8).

Heterotrimeric G proteins function as signal transducers from receptors in the cell membrane to intracellular effectors. They consist of three subunits (α, β, γ) and are classified into four subfamilies, Gα, Gβγ, and G12 (9). The α-subunits of Gα family G proteins, including Gαi, Gαs, transducin, and gustducin, are specifically ADP-ribosylated by pertussis toxin (PTX) to become unable to couple with GPCRs. Therefore, PTX serves as a useful tool to explore Gα-mediated signal transduction both in vitro and in vivo. The α- and/or βγ-subunits of Gαi and Gαs directly regulate adenylyl cyclase, phospholipase Cβ, phosphatidylinositol 3-kinase, and K+ channels. βγ-subunits released from Gαi indirectly stimulate ERK (9–11). In early embryonic brains, Gαi and Gγγ are highly expressed in the ventricular zone but their levels decrease with development (12, 13). In contrast, levels of Gαs and Gγγ, which are limited to the marginal zone, increase with the addition of newly generated neuronal cells from the ventricular zone (12, 14). In the adult brain, Gαs and Gγγ as well as Gαi1 and Gγγ3 are major isoforms of Gα proteins (15–18) but the expression of Gαi1 and Gγγ3 persists in the neural stem cells in the ventricular zone at the rostral part of lateral ventricle and progeny cells migrating toward the olfactory bulb (13). Therefore, the expression of Gαi2 (Gαi2βγγ3) seems to be consistently involved in neurogenesis, both in embryonic and postnatal brains.

To clarify the role of Gαi2 in the developing brain, we first injected PTX into the lateral ventricle of mouse embryos, and found suppression of neuroepithelial cell proliferation. Next, an in vitro survey of several Gαi-coupled receptor agonists revealed that endothelins (ETs) have potent mitogenic activity for neural progenitor cells via their action on ET-B receptors, and such an effect of ET was confirmed in vivo.

The abbreviations used are: FGF-2, fibroblast growth factor-2; G protein-coupled receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; ET, endothelin; E, embryonic day; LPA, lysophosphatidic acid; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; PBS, phosphate-buffered saline; SFM, serum-free medium.

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**EXPERIMENTAL PROCEDURES**

**Animal Preparation**—Each female mouse (Jcl: ICR) or rat (Sprague-Dawley) was mated with a male overnight and noon of the day to confirm the pregnancy was designated as embryonic day (E) 0. All the treatments of animals and the experimental procedures were conducted following the guidelines for care and use of laboratory animals, M, University School of Medicine and the Institute for Developmental Research, Aichi Human Service Center.

**Exo-utero Microinjection—**PTX or the vehicle was injected into the ventricles of developing mouse embryos at E14.5 with the exo-utero microinjection method, as previously reported (7,19). In short, pregnant mice were anesthetized with pentobarbital (50 mg/kg body weight) and injected with 1.4 mg of ritodrine (Sigma) dissolved in saline solution (0.1 ml) to relax the myometrium. The abdominal wall was cut, and the uterus gently pulled out of the peritoneal cavity. The uterine walls were cut longitudinally and 4–6 embryos were selected for injection, while other embryos were removed. By using glass pipettes (80–80-μm in diameter) under a binocular microscope, 1 μl of PTX (0.1 μg, Calbiochem) or the vehicle was injected into the ventricular cavity of the experimental and the control groups, respectively. After the uterus was returned to within the body, ~2 ml of warm Hank’s solution were gently injected, to prevent unexpected adhesions within the peritoneal cavity, and the abdominal wall was sutured. Then the animals were returned to the mouse room after confirmation of recovery from the anesthesia. The embryos were fixed at E18.5 for counting total cells in the cerebral cortices. To label proliferating cells, intraperitoneal injection of bromodeoxyuridine (BrdUrd, 50 mg/kg body weight) was performed on pregnant females at E16.5, and the animals were sacrificed and injection to collect embryos. For examination of the effect of ET, 1 μl of ET-3 (10 μM), BQ788 (1 mM), or vehicle was incubated for a further 3 days. Cells were washed twice with PBS, incubated with 0.2% trypsin for 15 min at 37 °C, and counted using a hematocytometer.

**Immunohistochemistry**—Sections of the parietal cortex (at the level of the suprachiasmatic nucleus) were photographed and developed at the final magnification of ×80. For each cerebral cortex, Nissl stain-positive cells and BrdUrd-positive cells were counted in ten different columnar zones (about 35-μm wide) extending from the ventricular lumen to the pial surface of the cerebral cortex. The choice of imaging areas and cell counts were performed blindly.

**Detection of Apoptotic Cells**—Sections close to those used for BrdUrd immunostaining were subjected to terminal deoxytransferase-mediated dUTP nick end labeling (TUNEL) assay, which was performed with an in situ Cell Death Detection Kit (Roche Diagnostics). Samples were analyzed by following immunostaining with peroxidase conjugated anti-fluorescein antibody and reaction with diaminobenzidine.

**Primary Culture of Neural Progenitor Cells of Rats**—Neural progenitor cells were cultured according to the method described previously (20) with modifications. Forebrains of rat embryos at E13.5 were dissected and dissociated by incubation with 0.2% trypsin for 15 min followed by repeated trituration. To remove contaminating fibroblasts tightly attached to the dishes, dissociated cells were seeded onto 100-mm uncoated culture dishes in the presence of 10% fetal bovine serum in serum-free medium (SFM). After 4 h incubation, cells floating or lightly attaching to fibroblasts were collected for seeding to culture dishes coated with poly2-hydroxy-ethyl methacrylate) (Sigma, 1.6 mg/cm2) (21) and cultured in SFM containing 20 ng/ml FGF-2 (Pepro Tech EC) and 2 μg/ml heparin (Sigma) for 6 days to generate proliferative progenitor neurospheres. Refreshment of a half volume of the medium was performed at 3 days. The SFM consisted of DMEM/F12 (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and nutrient additives including 0.01% bovine serum albumin (Fraction V), 30 nM selenium, 15 nM triiodo-thyronine, 40 nM biotin, 25 μg/ml apo-transferrin, 1 nM hydrocortisone, and 25 μg/ml insulin (all of these reagents were from Sigma). Neurospheres were dissociated by initial incubation with 0.05% trypsin for 10 min followed by repeated trituration. Dissociated cells were plated onto dishes precoated with 100 μg/ml poly-L-lysine (Sigma) plus or minus 10 μg/ml fibronectin (Nacalai Tesque), propagated in SFM with 10 ng/ml FGF-2 under an atmosphere of 5% CO2, 95% air and used for the various experiments.

**Immunofluorescence Staining**—Dissociated progenitor cells were plated on poly-L-lysine-coated coverslips within 35-mm dishes and cultured for 48 h in SFM in the presence of 10 ng/ml FGF-2 and then for 24 h in SFM without FGF-2. PTX (20 ng/ml) was added for the last 6 h. Exposure to agonists was for 24 h, with 0.2 μCi/well [methyl-3H]thymidine introduced for the last 6 h of the incubation at 37 °C. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), and 5% trichloroacetic acid was added for 20 min at 4 °C. The cells were then washed once with 5% trichloroacetic acid, and a mixture of 0.1 M NaOH, and 1% SDS was added for 10 min. Samples were transferred to scintillation vials, and the radioactivity was counted with a Beckman LS6000IC scintillation counter. The agonists and antagonists used were as follows: ET-1 and ET-3 (Peptide Institute), lysophosphatidic acid (LPA, Cayman Chemical), carbachol (Sigma), dopamine (Wako Pure Chemical Industries), U-69593 (Biomol Research Laboratories), BQ123 (Research Biochemicals International), BQ788 (Sigma), and U0126 (Calbiochem).

**Cell Proliferation**—Dissociated progenitor cells (4 × 105/dish) were plated to 35-mm dishes and cultured for 4 h in SFM in the presence of 10 ng/ml FGF-2 and then for 24 h in SFM without FGF-2. PTX (20 ng/ml), and inhibitors were added for the last 6 h and the last 30 min, respectively. At various times after addition of agonists, cells were washed twice with ice-cold PBS and lysed with 1% SDS in 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The amount of protein in each lysate was quantified using a micro bichinonic acid protein assay kit (Pierce), with bovine serum albumin as the standard. The lysates (15 μg of proteins) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted with rabbit polyclonal antibody to phosphorylated or unphosphorylated ERK1/2 (1:1000, Cell Signaling Technology). Immunostaining was achieved using a LAS-1000 (Fujifilm).

**Immunofluorescence for ERK**—Dissociated progenitor cells (5 × 105/dish) were plated to 35-mm dishes and cultured for 48 h in SFM in the presence of 10 ng/ml FGF-2 and then for 24 h in SFM without FGF-2. PTX (20 ng/ml), and inhibitors were added for the last 6 h and the last 30 min, respectively. At various times after addition of agonists, cells were stained with anti-GAP43 (rabbit, 22, G17, 10 μg/ml) (18), nestin (mouse, 1:2000, BD Pharmingen), β-tubulin type III (mouse, 1:400, Chemicon), and glial fibrillary acidic protein (mouse, 1:600, Sigma), and visualized with Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 568 anti-mouse IgG, Alexa Fluor 488 anti-mouse IgG, or Alexa Fluor 568 anti-rat IgG (1:400, Molecular Probes), for 1 h at room temperature. For differentiation, cells were transferred to culture dishes coated with poly(2-hydroxy-ethyl methacrylate) (Sigma, 1.6 mg/cm2) (21) and cultured in SFM containing 20 ng/ml FGF-2 (Pepro Tech EC) and 2 μg/ml heparin (Sigma) for 6 days to generate proliferative progenitor neurospheres. Refreshment with a half volume of the medium was performed at 3 days.
membranes or neural progenitor cells were solubilized in 20 mM Tris-HCl (pH 7.5) and sonicated and then centrifuged at 4°C for 30 min. The supernatant, referred to as the cholate extract, was incubated at 37°C for 60 min at 30°C in 20 mM Tris-HCl (pH 7.5), 2 μM [32P]NAD, 10 mM thymidine, 1 mM EDTA, and 1 mM iodoacetic acid and 1 mM ATP. The reaction was terminated by the addition of Laemmli sample buffer (23), then aliquots (1.5 μg of protein) were subjected to SDS-PAGE, with subsequent autoradiography using x-ray film (RX-U, Fujifilm). The levels of Gα2, Gαi2, and Gβ proteins in cholate extracts were determined by immunoblotting with antibodies to Gα2 (second panel), Gαi2 (third panel), and Gβ (bottom panel), respectively.

RESULTS

Effects of PTX on the Developing Mouse Brain—To block the function of Gα2 in the ventricular zone, PTX was injected into the lateral ventricle of mice at E14.5. When the embryos were fixed at E18.5, there was no significant decrease in body weight and crown-rump length of PTX-injected embryos compared to control embryos. A and B, embryos were injected with PTX or vehicle at E14.5 and fixed at E18.5, and sections were stained with cresyl violet. The cerebral cortex of a PTX-injected embryo is thinner than that of a control embryo (A). The cell numbers in 10 columnar areas of the cerebral cortex were counted and are shown as means ± S.E. of results from 7 control and 5 PTX-treated embryos (3 litters). *, p < 0.05 (B).

FIG. 2. PTX-injected embryos have a thinner cerebral cortex as a result of fewer BrdU-positive cells without increase in cell death. A and B, embryos were injected with PTX or vehicle at E14.5 and fixed at E18.5, and sections were stained with cresyl violet. The cerebral cortex of a PTX-injected embryo is thinner than that of a control embryo (A). The cell numbers in 10 columnar areas of the cerebral cortex were counted and are shown as means ± S.E. of results from 7 control and 5 PTX-treated embryos (3 litters). *, p < 0.05 (B).

EVALUATION OF PTX-MEDIANATED ADP-RIBOSYLATION OF G PROTEINS IN THE CEREBRAL CORTEX OF MOUSE BRAINS OR CULTURED CELLS—Two days after injection of PTX or vehicle solution at E14.5, embryos were collected from dams and whole brains without the cerebellum were dissected. Cultured neural progenitor cells were incubated with 20 ng/ml PTX at 37°C for 6 h and washed with PBS. These samples were kept frozen at −80°C until analysis. Frozen brains were homogenized at 0°C in a Potter-Elvehjem homogenizer in 20 volumes (v/w) of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl, and centrifuged at 4°C for 20,000 × g for 20 min to obtain the membrane fraction. The brain membranes or neural progenitor cells were solubilized in 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 100 mM NaCl, and 1% sodium cholate, sonicated and then centrifuged at 4°C at 100,000 × g for 40 min. The supernatant, referred to as the cholate extract, was incubated for 60 min at 30°C in 20 mM Tris-HCl (pH 7.5), 2 μM [32P]NAD, 10 mM thymidine, 1 mM EDTA, and 1 mM iodoacetic acid and 1 mM ATP. The reaction was terminated by the addition of Laemmli sample buffer (23), then aliquots (1.5 μg of protein) were subjected to SDS-PAGE, with subsequent autoradiography using x-ray film (RX-U, Fujifilm). The levels of Gα2, Gαi2, and Gβ proteins in cholate extracts were determined by immunoblotting with antibodies to Gα2 (22), Gαi2 (16), and Gβ (22), respectively.

Statistical Analysis—All results are given as means ± S.E. values. The significance of group differences was analyzed with the Student’s t test for single comparisons, and with one-way analysis of variance followed by the Bonferroni correction for multiple comparisons. A p value of < 0.05 was considered significant.
with controls (Fig. 1, A and C). However, PTX-injected embryos showed a decrease in the head vault compared with those of controls (Fig. 1A) and the cerebral cortices taken from the PTX-injected embryos were smaller than those of control embryos (Fig. 1, B and C). When they were taken out at E19.5 (full term), most PTX-injected mice could hardly start respiration and died. To confirm the effect of PTX, ADP-riboylation of endogenous G\textsubscript{i2} proteins was evaluated in cholate extracts from PTX- or vehicle-injected brains at E16.5 using \[^{32}P\]NAD, and compared the results with those for FGF-2, known to be a major mitogen which binds tyrosine kinase receptors. Among other PTX-sensitive G\textsubscript{i} subunits, G\textsubscript{i2} was expressed at a low level in these cells, but G\textsubscript{i2} and G\textsubscript{i3} were not detectable by immunoblot analysis (data not shown). After withdrawal of FGF-2 for 7 days, many cells expressed neuron-specific antigen (\(\beta\)-tubulin type III) or an astrocyte marker (glial fibrillary acidic protein (GFAP)), indicating that they retained properties of undifferentiated cells (Fig. 3A). These cells also expressed G\textsubscript{\alpha}\textsubscript{S} and G\textsubscript{\alpha}\textsubscript{B}, as observed previously in the ventricular zone of embryonic brain (Fig. 3A) (12).

Among other PTX-sensitive G\textsubscript{\alpha} subunits, G\textsubscript{\alpha} was expressed at a low level in these cells, but G\textsubscript{i2} and G\textsubscript{i3} were not detectable by immunoblot analysis (data not shown). After withdrawal of FGF-2 for 7 days, many cells expressed neuron-specific antigen (\(\beta\)-tubulin type III) or an astrocyte marker (glial fibrillary acidic protein (GFAP)), indicating that they retained properties of undifferentiated cells (Fig. 3A). These cells also expressed G\textsubscript{\alpha}\textsubscript{S} and G\textsubscript{\alpha}\textsubscript{B}, as observed previously in the ventricular zone of embryonic brain (Fig. 3A) (12).

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Immunoblot analyses of the same extracts revealed levels of total G\textsubscript{\alpha} and G\textsubscript{i} proteins in PTX-injected brains to be somewhat decreased (G\textsubscript{\alpha}, 72 ± 3% of the control; G\textsubscript{i}, 81 ± 4% of the control, n = 6) than those from control brains, indicating that a large proportion of G\textsubscript{\alpha} proteins had already been ADP-ribosylated by the injection of PTX at E14.5 (Fig. 1D). Immunoblot analyses of the same extracts revealed levels of total G\textsubscript{\alpha} and G\textsubscript{i} proteins in PTX-injected brains to be somewhat decreased (G\textsubscript{\alpha}, 72 ± 3% of the control; G\textsubscript{i}, 81 ± 4% of the control, n = 6) than those from control brains, indicating that a large proportion of G\textsubscript{\alpha} proteins had already been ADP-ribosylated by the injection of PTX at E14.5 (Fig. 1D). Immunoblot analyses of the same extracts revealed levels of total G\textsubscript{\alpha} and G\textsubscript{i} proteins in PTX-injected brains to be somewhat decreased (G\textsubscript{\alpha}, 72 ± 3% of the control; G\textsubscript{i}, 81 ± 4% of the control, n = 6) than those from control brains, indicating that a large proportion of G\textsubscript{\alpha} proteins had already been ADP-ribosylated by the injection of PTX at E14.5 (Fig. 1D).

In contrast, the levels of G\textsubscript{\beta} (Fig. 1D, 101 ± 4% of the control, n = 6) and PTX-insensitive G\textsubscript{\alpha}11 proteins (101 ± 9% of the control, n = 6) were unchanged by PTX treatment.

Brains of PTX-injected Embryos Have Thinner Cerebral Cortices than Those of Control Embryos—Histological studies revealed that the cerebral cortex from PTX-injected embryos at E18.5 was thinner than those from controls (Fig. 2A) and the total cell number in this zone was significantly reduced (Fig. 2B). To label proliferating cells, BrdUrd was administrated 2 days after PTX injection, and embryos were fixed 2 h later. Immunostaining with an antibody against BrdUrd showed a decreased number of BrdUrd-positive cells in the cerebral cortex of PTX-injected embryos (Fig. 2, C and D). TUNEL staining of close sections revealed that injection of PTX did not significantly increase cell death in the cerebral cortex (Fig. 2, E and F). Moreover, PTX seems not to affect the migration of neural cells, because there was no significant decrease in the number of neural cells labeled with BrdUrd before PTX injection and migrated from the ventricular zone into the cortical plate (control, 39.8 ± 8.7; PTX, 35.5 ± 9.4 in the area shown by box in Fig. 2F, n = 4). These results suggest that G\textsubscript{\alpha} mediates signaling from GPCRs to maintain proliferation of neural progenitor cells in the developing brain. To investigate which receptors are coupled to G\textsubscript{\alpha} to stimulate neurogenesis, we next performed in vitro experiments using neural progenitor cells dissociated from rat embryonic brains.

**Characterization of Cultured Neural Progenitor Cells—**Proliferative progenitor neurospheres were generated from fore brains of E13.5 rats, dissociated, and cultured in the presence of FGF-2. The majority of cells were nestin-positive, indicating that they retained properties of undifferentiated cells (Fig. 3A). These cells also expressed G\textsubscript{\alpha} and G\textsubscript{i}, as observed previously in the ventricular zone of embryonic brain (Fig. 3A) (12). Among other PTX-sensitive G\textsubscript{\alpha} subunits, G\textsubscript{\alpha} was expressed at a low level in these cells, but G\textsubscript{i2} and G\textsubscript{i3} were not detectable by immunoblot analysis (data not shown). After withdrawal of FGF-2 for 7 days, many cells expressed neuron-specific antigen (\(\beta\)-tubulin type III) or an astrocyte marker (glial fibrillary acidic protein (GFAP)), indicating that they retained properties of undifferentiated cells (Fig. 3A). These cells also expressed G\textsubscript{\alpha} and G\textsubscript{i}, as observed previously in the ventricular zone of embryonic brain (Fig. 3A) (12).

**ETs Increase Proliferation of Neural Progenitor Cells through G\textsubscript{\alpha}**—Several G\textsubscript{\alpha}-coupled receptors have been found to be expressed in the ventricular zone of developing brains, including ET-B (24), LPA (25), muscarinic acetylcholine (6), D\textsubscript{3} dopamine (26), and \(\kappa\)-opioid receptors (27). Therefore, we examined whether agonists for these receptors might stimulate the incorporation of \[^{3}H\]thymidine in neural progenitor cells, and compared the results with those for FGF-2, known to be a major mitogen which binds tyrosine kinase receptors. Among the agonists for G\textsubscript{\alpha}-coupled receptors, ET-3 was most effective,
while LPA also significantly increased the incorporation of \(^{[3}H\)thymidine (Fig. 4). However, such stimulatory effects were not observed with the other agonists such as carbachol, dopamine, and U-69593 (an agonist for the \(/H9260\)-opioid receptor). In these experiments, we also found that ET-3-stimulated \(^{[3}H\)thymidine incorporation was greatly enhanced in the presence of fibronectin, but the FGF-2-mediated effect was not so augmented (Fig. 4). Laminin, another extracellular matrix component, was similarly effective (data not shown). Therefore, cell culture was carried out on dishes coated with poly-L-lysine plus fibronectin unless otherwise described.

ET-1 and ET-3 revealed a similar dose-dependence for stimulation of \(^{[3}H\)thymidine incorporation with submaximal effects at 1 nM (Fig. 5A). The stimulatory effects were blocked by an ET-B receptor antagonist BQ788, but not by an ET-A receptor antagonist BQ123 (28) (Fig. 5B). These results indicate that the effects of ETs are mediated by the ET-B receptor. Because ET receptors are known to couple not only with \(Gi\) but also with \(Gq/11\) and \(G13\) (29, 30), we next examined whether \(Gi\) is indeed involved in stimulation of DNA synthesis. PTX treatment of neural progenitor cells partially decreased ET-3-enhanced \(^{[3}H\)thymidine incorporation (Fig. 5C), whereas the toxin completely ADP-ribosylated endogenous \(G_{iq}\) proteins (Fig. 5C, inset), suggesting that both \(G_{iq}\) and PTX-insensitive \(Gp\) proteins mediate ET-stimulated DNA synthesis. Pertussis toxin did not influence FGF-2-stimulated \(^{[3}H\)thymidine incorporation, suggesting that \(G_{iq}\) is not involved in FGF-2-mediated DNA synthesis (Fig. 5C). To determine whether ETs actually increase cell proliferation, cell numbers were counted 3 days after stimulation. As shown in Fig. 6, ET-3 significantly increased cell proliferation of neural progenitor cells.

**Fig. 5. Effects of ETs and inhibitors on \(^{[3}H\)thymidine incorporation in cultured neural progenitor cells.** A, effects of increasing concentrations of ET-1 and ET-3. B, effects of antagonists for ET receptors on ET-stimulated \(^{[3}H\)thymidine incorporation. Antagonists were added 30 min before addition of agonists, the respective concentrations being 5 \(\mu M\) and 10 nM. C, effects of PTX and U0126, an inhibitor of MAP kinase, on ET-3 and FGF-2-stimulated \(^{[3}H\)thymidine incorporation in neural progenitor cells. PTX (20 ng/ml) and U0126 (2 \(\mu M\)) were added to cells 6 h and 30 min before stimulation by 10 nM ET-3 and 10 ng/ml FGF-2. Inset, autoradiogram of \(^{[3}P\)ADP-ribosylated \(G_{iq}\) from cells that had not been treated or had been pretreated with PTX. Values are means ± S.E. from four separate experiments performed in duplicate. *, \(p < 0.05\).

**Fig. 6. Effects of ET-3 on proliferation of neural progenitor cells.** Dissociated progenitor cells (4 \(\times 10^5\)) were plated onto 35-mm dishes coated with poly-L-lysine plus or minus fibronectin and cultured for 24 h in SFM in the presence of 10 ng/ml FGF-2 and then for 24 h in SFM without FGF-2. PTX (20 ng/ml) was added for the last 6 h. ET-3 (10 nM) or FGF-2 (10 ng/ml) was added to cells, incubated for 3 days, and cells were counted. Values are means ± S.E. from four separate experiments performed in triplicate. *, \(p < 0.05\).

**Fig. 7. Effects of intraventricular administration of an ET-B receptor agonist or antagonist on numbers of BrdUrd-positive cells.** Embryos were injected with ET-3, BQ788 or vehicle at E14.5, followed by BrdUrd labeling at E15.5 and fixed 2 h later, and sections were stained with anti-BrdUrd antibodies. The numbers of BrdUrd-positive cells in 10 columnar areas of the cerebral cortex were counted and are shown as means ± S.E. of results from 4 control and 4 ET-3-treated embryos (B) or from 5 control and 5 BQ788-treated embryos (D). *, \(p < 0.05\). Bar, 50 \(\mu M\). V, lumen of ventricular cavity.
numbers in the presence of fibronectin but its effect was small in its absence. ET-stimulated proliferation was partially inhibited by pretreatment with PTX, indicating that ET stimulates cell proliferation at least in part via $G_{i2}$. PTX did not inhibit FGF-2-stimulated proliferation.

To examine whether ET is indeed acting as a mitogen for the progenitors in the ventricular region, an agonist and an antagonist for the ET-B receptor were injected into the ventricle of mice at E14.5, followed by BrdUrd labeling. ET-3 injection increased a number of BrdUrd-positive cells in the cerebral cortex (Fig. 7, A and B), while BQ788 injection decreased it (Fig. 7, C and D).

ET-3 Stimulates Phosphorylation of ERK through $G_{i2}$—Since $G_i$ is known to stimulate the MAPK cascade that induces DNA synthesis and cell proliferation (11), we determined whether this pathway is involved in ET-stimulated proliferation of neural progenitor cells. ET-stimulated DNA synthesis was markedly inhibited by U0126, an inhibitor of MAPK kinase (Fig. 5C). Next we investigated the abilities of ET-3 and FGF-2 to activate ERK by measuring phosphorylation of ERK1/2. Because ERK2 is a major form in neural progenitor cells and its phosphorylation is much clearer than that of ERK1, phosphorylation of ERK2 was quantified in the present study. ET-3 rapidly increased phosphorylation of ERK2, maximally between 5 and 10 min, and then the levels gradually decreased. In contrast, FGF-2 slowly increased phosphorylation of ERK2 and a high level was sustained for a long time (Fig. 8A). Fibronectin did not significantly enhance the maximal stimulation of ERK phosphorylation by both ET-3 and FGF-2. However, ET-stimulated phosphorylation at 30 min was significantly higher in the presence of fibronectin than that in its absence, suggesting that fibronectin signaling may sustain higher levels of ET-induced ERK phosphorylation (Fig. 8A). ET-3-stimulated phosphorylation of ERK2 was diminished by treatment with PTX and U0126 (Fig. 8B), an observation in good agreement with the results obtained for DNA synthesis (Fig. 5C). PTX did not affect FGF-2-increased ERK phosphorylation (data not shown). The stimulatory effect of ET-3 on ERK2 phosphorylation was also blocked by the ET-B receptor antagonist BQ788, but not by the ET-A receptor antagonist BQ123 (Fig. 8C).

Fate of ET-expanded Cells—We next determined the fate of the proliferating cells after ET treatment. Neural progenitor cells were cultured with ET-3 and BrdUrd, which were then removed from the medium to continue incubation for 3 or 6 days. Cells were double-stained with antibodies to BrdUrd and various cell marker proteins including $\alpha$-smooth muscle actin, because recent studies showed neural progenitor cells to differentiate into smooth muscle cells (31, 32). Fig. 9 shows that ET-expanded cells were destined to become not only neurons and astrocytes but also smooth muscle cells. Smooth muscle cells were also observed when neural progenitor cells were cultured for 4 days in the presence of ET during the overall incubation (data not shown).

DISCUSSION

We have previously shown that $G_{i2}$ and $G_{i10}$ are exclusively expressed in areas where neurons are actively generated in the embryonic and postnatal brains of rats, while other PTX-sensitive G proteins are abundant in differentiated neuron-rich areas (12, 13). In the present study, we investigated the role of $G_{i2}$ by injection of PTX into the lateral ventricle of developing mouse embryos and found that blockade of the $G_{i2}$ pathway leads to decreased levels of proliferation of neuroepithelial cells in the ventricular zone. The injection of PTX effectively ADP-ribosylated the endogenous $G_{i10}$ in the brains of mouse embryos. This is in striking contrast to the results obtained with PTX-injected adult rat brains in which most $G_{i10}$ proteins were not ADP-ribosylated because of limited penetration of PTX into the brain tissues from the ventricular cavity (33). Thus, injection of PTX into the ventricular cavity of embryos seems to be useful to investigate the roles of $G_i$ family G proteins in the developing brain.

Evaluation of $G_i$-coupled receptors for their effects on the incorporation of $[^3H]$thymidine in cultured neural progenitor cells here revealed ET to be the most effective of the agonists studied. DNA synthesis was stimulated also by LPA to some degree, but not by other agonists, including carbachol, in disagreement with an earlier report (6). Although the reason for the discrepancy is not clear, differences in the procedures for cell preparation (for example, generation of neurospheres)
and double-stained with antibodies to BrdUrd (red/H9252 red/H9262), 50 Bar onto coverslips coated with poly-L-lysine plus fibronectin and cultured after removal of the agonist. for 48 h in SFM in the presence of 10 nM ET-3, with 10

might have influenced the response. Although ETs were originally found to be potent vasoactive peptides secreted from the endothelial cells of blood vessels (34, 35), later studies show that they have mitogenic effects on various cells, including vascular smooth muscle cells, fibroblasts (29), astrocytes (36), and neural crest cells (37). To our knowledge, this is the first report on the propagation of neural progenitor cells from cerebral cortex by ETs. In the present study, ET-1 and ET-3 similarly stimulated the levels of [3H]thymidine incorporation and this stimulatory effect was blocked by an ET-B receptor antagonist, but not by an ET-A receptor antagonist, indicating mediation by ET-B receptors. The ET-stimulated DNA synthesis observed in cell culture experiments was also confirmed in vivo by the intraventricular administration of an agonist and an antagonist for ET-B receptors in developing brains.

ET-B receptors are expressed in the ventricular zone in embryonic brains and the subventricular zone in adult brains (24). Similar localization of ET-B receptors and Gaαq/Gqγ6 proteins (12, 13) suggests a functional link of these molecules in neurogenesis. Where do ETs come from in the developing brain? One possible origin is the capillary endothelial cells, since the ET-1 precursor, preproendothelin-1, is expressed in capillary endothelial cells sprouting out of meningeal vessels to penetrate into developing mouse brains at E11.5 (38). The neuroepithelial cells of the brain vesicle have radial fibers that contact with or encircle blood vessels (39), suggesting easy access of ETs secreted from capillary endothelial cells to ET-B receptors in the neuroepithelial cells. Moreover, neurogenesis occurs within an angiogenic niche in the adult hippocampus of rats (40) or in a coordinated fashion with angiogenesis in the adult songbird brain (41). Recently, a growth factor secreted from the endothelial cells of the blood vessels, vascular endothelial growth factor, has also been reported to stimulate neurogenesis in vitro and in vivo (42). A second source of ETs may be the choroid plexus, because higher concentrations of human ETs are reported in the cerebrospinal fluid than the plasma (29).

Previous studies showed that ET-B receptors couple not only with G, but also Gaq11 and G13 (29, 30). ET-stimulated proliferation of neuroepithelial cells was partially blocked by PTX in the present study, suggesting it to be mediated by both G, and other G proteins. Stimulation of ET-B receptors increased phosphorylation of ERK in the MAPK cascade, which is known to be an important pathway between G proteins and DNA synthesis. ET-stimulated ERK phosphorylation was also partially inhibited by PTX, indicating the MAPK cascade to be downstream from Gαq. It is generally accepted that activation of ERK by Gαq is due to Gβγ (11), and Gβγ seems to mediate this signaling pathway in the embryonic brain.

In the present study, we found that ET-induced DNA synthesis and cell proliferation were enhanced by fibronectin. The distribution of fibronectin changes in coordination with the development of the cerebral cortex (43). Fibronectin production begins first in the ventricular zone prior to formation of the cortical plate, and its levels decline gradually afterward. The second wave of fibronectin generation begins soon after cortical plate formation, with expression levels high in the intermediate zone dispersed with migrating neurons, and in the upper cortical plate. From these observations, Sheppard et al. (43) speculated that fibronectin might be involved initially in supporting the cell division and fate determination that takes place in the ventricular zone and later in assisting cell migration in the intermediate zone. The stimulating effects of fibronectin on mitogenic activity of neural progenitor cells in the present study appear consistent with this hypothesis regarding the early stage. Fibronectin did not markedly influence ET-stimulated ERK phosphorylation, in contrast to its potent effect on DNA synthesis, though it seemed to help maintain ERK phosphorylation at higher levels for a relatively long time. Previous reports showed integrin regulation of GPCR signaling to ERK and DNA synthesis. ET-1 was shown to induce DNA synthesis in primary astrocytes by stimulating the ERK pathway via PTX-insensitive G proteins, and ET-stimulated DNA synthesis was adhesion-dependent, whereas ERK activation was independent of integrin signaling, suggesting at least two distinct pathways leading to DNA synthesis (44). In contrast, P2Y receptor signaling to ERK is highly dependent on integrin-mediated cell anchorage in endothelial cells (45). On the other hand, the fact that ET significantly increased cell numbers only in the presence of fibronectin may suggest fibronectin to be acting as a survival factor. Actually, more cells survived in the presence of fibronectin, when neural progenitor cells were cultured with ET for 1 day and then without ET for 6 days in the presence or absence of fibronectin (data not shown).

It is well known that neural progenitor cells can differentiate either into neurons or glial cells and actually both PGE2- and ET-expanded cells differentiate into both of these after removal
of agonists. In addition, recent reports demonstrated that these progenitor cells could also differentiate into smooth muscle cells (31, 32). In the present study, we showed ET-expanded progenitor cells to express smooth muscle actin, indicating differentiation into smooth muscle cells. Such differentiation was also observed in the presence of ET during the overall incubation (data not shown), suggesting that ET enhances proliferation and simultaneously induces differentiation into smooth muscle cells in the population of neural progenitor cells. These findings suggest that ET cannot maintain progenitor cells in an undifferentiated state, unlike FGF-2. In addition, ET did not stimulate in proliferation in neurospheres, probably due to non-attachment to dishes (data not shown), indicating that neural progenitor cells cannot be maintained in vitro by only ETs.

In the present study, we showed proliferation of neural progenitor cells to be stimulated by ETs and FGF-2, mediated by GPCR and receptor tyrosine kinase, respectively. A previous study indicated that PTX blocked FGF-2-mediated activation of G protein-coupled receptor (GPCR) and receptor tyrosine kinase, respectively. A previous study demonstrated that ETs and FGF-2, mediated by different signaling pathways, may be coordinated during embryonic neurogenesis.

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Gi2 Signaling Enhances Proliferation of Neural Progenitor Cells in the Developing Brain

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