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# Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells

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**Abstract**

Sphingosine-1-phosphate (S1P) is a lipid mediator that exerts multiple cellular functions through activation of G-protein-coupled receptors. Although the role of S1P on angiogenesis is well established, its role in neurogenesis is unknown. We examined the effects of S1P on G-protein activation in brain sections of rat embryo and on neural progenitor cells in culture. Intense S1P-stimulated [$^{35}$S]GTPγS labeling was observed as early as E15 in the neuroepithelium and differentiating fields throughout the brain, suggesting that functional S1P receptors are expressed in brain areas with active neurogenesis. mRNA transcripts for several S1P receptor subtypes ($S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$) were expressed in neural progenitor cells prepared from embryonic rat hippocampus. S1P induced phosphorylation of extracellular signal-regulated kinase (ERK) and proliferation of neural progenitor cells as determined by BrdU incorporation in a pertussis toxin-sensitive manner. These effects were prevented by the ERK signaling inhibitor U0126. S1P augmented telomerase activity in neural progenitor cells with similar potency as that of FGF-2. Furthermore, S1P induced cell-cell aggregation. This morphological change was transient and prevented by Y-27632, an inhibitor of Rho-associated kinase. These results suggest that S1P plays a pleiotropic role in neurogenesis via pathways involving S1P receptors, MAP kinases and Rho kinase.

**Keywords:** MAP kinase, neural progenitor cells, neurogenesis, Rho-associated kinase, sphingolipids, telomerase.
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Neurons and glia in the mature CNS originate from multipotent neural stem or precursor cells in the ventricular and subventricular zones of the fetal brain and spinal cord. Recent studies have also demonstrated that neurons can be born within specific regions of adult brain (Eriksson *et al*. 1998; Gage 2000; Donovan and Gearhart 2001; Gould and Gross 2002).

The role of fibroblast growth factor (FGF)-2 and epidermal growth factor in the proliferation of neural stem cells and progenitor cells is well established (Ray *et al*. 1993; Gritti *et al*. 1996; Weiss *et al*. 1996; Yoshimura *et al*. 2001) but other growth factors, hormones and cytokines may also be involved (Cameron and Gould 1994; Arsenijevic *et al*. 2001; Erlandsson *et al*. 2001; Shimazaki *et al*. 2001; Jin *et al*. 2002). Neurotransmitters may also regulate proliferation and differentiation of these cells (Ma *et al*. 2000; Li *et al*. 2001).

The sphingolipid sphingosine-1-phosphate (S1P) affects multiple cell processes (Pyne and Pyne 2000; Yatomi *et al*. 2001; Spiegel and Milstien 2002). In particular, S1P induces proliferation, morphogenesis, migration of vascular endothelial cells (Lee *et al*. 1999) and angiogenesis (Liu *et al*. 2000; English *et al*. 2002). A family of cell surface G-protein-coupled receptors (GPCRs) termed $S1P_{1\text{-}5}$ (previously $Edg_{1,5,3,6,8}$) mediates most of the biological actions of S1P. Another related bioactive lipid, lysophosphatidic acid (LPA), activates $LPA_{1\text{-}3}$ receptors (previously termed $Edg_{2,4,7}$; Spiegel and Milstien 2000; Chun *et al*. 2002; Kluk and Hla 2002). $LPA_1$ receptors are expressed in embryonic

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*Abbreviations used*: BrdU, bromodeoxyuridine; CREB, cAMP-responsive element binding protein; DH-S1P, dihydrosphingosine-1-phosphate; Egr-1, early growth response-1; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GPCR, G-protein-coupled receptors; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; p90 RSK, p90 ribosomal kinase; PBS, phosphate-buffered saline; S1P, sphingosine-1-phosphate; TBS, Tris-buffered saline; TRAP, telomeric repeat amplification protocol.

  

cortical ventricular zone and may be involved in neurogenesis (Hecht *et al*. 1996).

There is evidence that S1P plays a role in the adult as well as developing CNS. $S1P_2$ receptors are expressed in young, differentiating neuronal cell bodies and axons and are found in neuronal cell bodies during early stages of differentiation and in growing axons (MacLennan *et al*. 2001). Using [$^{35}$S]GTPγS autoradiography, we have shown that S1P activates G proteins in the subventricular zones of newborn rats (Waeber and Chiu 1999).

We have now found that S1P activates Pertussis toxin-sensitive G-protein coupled receptors in proliferative areas as early as E15. To characterize the signaling pathway of these receptors and their potential role in neurogenesis, we examined the actions of S1P on neural progenitor cells cultured from embryonic rat hippocampus. We found that S1P stimulates the proliferation of neural progenitor cells via ERK activation in a Pertussis toxin-sensitive manner. S1P also induces morphological changes by activating Rho associated kinases. These results suggest that S1P plays an important role during brain development.

## Materials and methods

### [$^{35}$S]GTPγS autoradiography

Brains from E15, E16, E18 and E21 Sprague-Dawley rat embryos ($n \geq 5$ for each age) were frozen in isopentane cooled to −40°C with dry ice. Sections, 14 μm thick, were cut using a cryostat-microtome (Microm HM505E) and thaw mounted onto fully frosted glass slides coated with gelatin. Sections were stored at −80°C for less than 3 weeks. Agonist-stimulated [$^{35}$S]GTPγS binding was visualized as previously described (Waeber and Chiu 1999; Happe *et al*. 2001) with minor modifications. Sections were thawed to room temperature 15 min before the experiment and incubated for 15 min at room temperature (20–24°C) )in 50 mM glycylglycine buffer (pH 7.4) containing 150 mM NaCl and 1 mM EGTA, and for a further 30 min in the same buffer supplemented with 3 mM $MgCl_2$, 0.2 mM dithiothreitol, and 2 mM guanosine 5′-diphosphate (GDP). In order to observe agonist-induced binding, sections were incubated in the appropriate concentration of agonist for 60 min at 30°C in buffer containing 2 mM GDP, 100 nM DPCPX (to block adenosine $A_1$ receptors stimulated by endogenously formed adenosine; Laitinen 1999) and 0.1 nM [$^{35}$S]GTPγS. Slides were then washed for 5 min twice in ice-cold buffer (without GDP), dipped briefly in ice-cold deionized water, dried under a stream of cold air, and exposed to Kodak Bio-Max film.

### Culture of neural progenitor cells

Rat hippocampal neural progenitor cells were cultured according to the method of Amoureux *et al*. (2000) with modifications. Hippocampi were dissected from E17 Sprague-Dawley rat embryos (Charles River Laboratories, Wilmington, MA, USA) into phosphate-buffered saline (PBS) and cells were mechanically dissociated. After centrifugation, cells were plated on poly-D-lysine-coated 100-mm cell culture dishes in Neurobasal medium containing 2% B27 supplement (Life Technologies, Gaithersburg, MD, USA) and 10 ng/mL FGF-2 (R & D Systems, Minneapolis, MN, USA). Five days later, cells were detached with trypsin and re-plated. These cells were used for experiments after two to four passages. Cortical neuronal culture was prepared in neurobasal medium containing 2% B27 supplement and used for experiments after maturation for 11–14 days *in vitro*.

### Immunocytochemistry

For differentiation, culture medium was changed to DMEM/F12 medium containing 1% N2 supplement (Life Technologies) and 100 ng/mL retinoic acid. Fetal bovine serum (1 or 10%, Life Technologies) was also added. Cells were then cultured for 7 days and fixed with 4% paraformaldehyde in PBS for immunocytochemistry. The following primary antibodies were used: mouse monoclonal anti-nestin antibody (used at a final dilution of 1 : 500, BD PharMingen, San Diego, CA, USA), mouse monoclonal anti β-tubulin isotype 3 (TuJ1) antibody (1 : 500, Berkeley Antibody Industry, Richmond, CA, USA), mouse monoclonal anti-O4 antibody (1 : 40, Roche Diagnostics, Indianapolis, IN, USA), and Cy3-conjugated anti-glial fibrillary acidic protein (GFAP) antibody (1 : 1000, Sigma-Aldrich, St Louis, MO, USA). Nestin antibody was revealed using biotin-avidin-horseradish peroxidase Vectastain system (Vector Laboratories, Burlingame, CA, USA). For double staining, cells were incubated with anti-TuJ1 or O4 antibodies followed by biotinylated anti-mouse IgG and avidin-Cy2 conjugate (Jackson ImmunoResearch, West Grove, PA, USA), after which, cells were incubated with Cy3-conjugated anti-GFAP antibody.

### Western blotting

For differentiation study, cells were plated on 100-mm dishes at $2 \times 10^6$ cells/dish and differentiation was performed as described for immunocytochemistry. Cells were cultured further for 1–7 days and cell extracts were prepared. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) (30 μg protein each) and western blotting were performed using standard methods. Antibodies used were the same as for immunocytochemistry, except anti β-actin antibody (1 : 2000) from Sigma, anti-microtubule-associated protein (MAP2) antibody (1 : 500) from Chemicon (Temecula, CA, USA), and anti-p27 antibody (1 : 200) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### RT-PCR

Neural progenitor cells were grown in FGF-2-containing medium and differentiation was induced as described above. These cells and cortical primary cultured neurons were harvested from 60-mm dishes and total RNA was isolated with an RNeasy$^{TM.}$ kit (Qiagen, Valencia, CA, USA). Total RNA (1 μg) was reverse-transcribed with AMV reverse transcriptase XL (Life Sciences, St Petersburg, FL, USA) and amplified by PCR with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: 5′-ATGGTGTCCTCCACCAGCATCCC-3′ (rat $S1P_1$ sense), 5′-TTAAGAAGAAGAATTGACGTTTCC-3′ (rat $S1P_1$ antisense), 5′-ATGGGCGGTTTATACTCAGAGT-3′ (rat $S1P_2$ sense), 5′-TCAGACCACTGTGTTGCCCTC-3′ (rat $S1P_2$ antisense), 5′-CGGCATAGCCTACAAGGTCA-3′ (rat $S1P_3$ sense), 5′-GATCACTACGGTCCGCAGAA-3′ (rat $S1P_3$ antisense), 5′-ATCTGTGCGCTCTATGCAAG-3′ (rat $S1P_5$ sense), 5′-TCTCGGTTGGTGAAGGTGTA-3′ (rat $S1P_5$ antisense), 5′-GCGGGGCGGTG-

CGTGACTAC-3′ (rat nestin sense), 5′-AGGCAAGGGGGAAGA-GAAGGATGT-3′ (rat nestin antisense), 5′-CGTAAAGACCTC-TATGCCAA-3′ (rat β-actin sense), 5′-AGCCATGCCAAATGTCT-CAT (rat β-actin antisense), 5′-CCTACAGGCCCAGCTACACT-3′ (rat GPR 3 sense), 5′-CCACCAGATGGTTCTTGGAGAG-3′ (rat GPR 3 antisense), 5′-GGTTGCTGCCCGTTCTAGGCTGG-3′ (rat GPR 6 sense), 5′- GGTCCTCTTGGCTACCCACCAC-3′ (rat GPR 6 antisense), 5′-GCCGTCGTGGTCCTTATCATCTTC-3′ (rat GPR 12 sense), 5′- ATTGTACGTGGCGGGCAGGAGGGTG-3′ (rat GPR 12 antisense), 5′-GGCAGTGAACAGCACAGTGGTG-3′ (rat GPR 63 sense), 5′-CCCAGGAGACTGCAATGAGAACC-3′ (rat GPR 63 antisense), 5′-AATCCTCAAGGGGAGCCGAGCGA-ACA-3′ (Egr-1 sense), 5′-TGCGGATGTGGGTGGTAAGGT-3′ (Egr-1 antisense), 5′-GATCCCAAGCGGCTCTAC-3′ (FGF-2 sense), 5′-GGACTCCAGGCGTTCAAAG-3′ (FGF-2 antisense). The conditions of the PCR reactions were 32 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (S1Ps, GPRs, Egr-1 and FGF-2); 27 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (nestin); 25 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min (β-actin); followed by 72°C for 7 min. Amplified DNA fragments were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide.

### Mitogen-activated protein (MAP) kinase activation

Cells were pre-treated with FGF-2-free medium for 3 h in the presence or absence of 10 μM U0126 (BioMol, Plymouth Meeting, PA, USA) or 300 ng/mL Pertussis toxin (Calbiochem, San Diego, CA, USA) and then treated with S1P for 10 min. S1P (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in ethanol at 20 mM and then suspended in fatty acid-free BSA (Roche, 4 mg/mL) solution at a concentration of 1 mM. After treatment, cells were washed with Tris-buffered saline (TBS) and lysed with Laemmli's sample buffer. Protein samples were separated by SDS–PAGE and protein band was detected by western blotting. The following antibodies were used: rabbit anti-phospho-p44/42 MAP kinase (ERK, Thr202/Tyr204) antibody, rabbit anti-p44/42 MAP kinase (ERK) antibody, rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, rabbit anti-phospho-p90 ribosomal kinase (p90 RSK, Ser380) antibody, rabbit anti-phospho-cAMP-responsive element binding protein (CREB, Ser133) antibody, rabbit anti-early growth response-1 (Egr-1) antibody. Antibodies were from Cell Signaling Technology Inc. (Beverly, MA, USA) except for anti-Egr-1 from Santa Cruz Biotechnology. Quantification of proteins was done by densitometric analysis of chemiluminescence-exposed films, using the NIH Image software (version 1.61).

### Proliferation assay

Cells were pre-treated with FGF-2-free medium for 3 h in the presence or absence of U0126 (10 μM) or Pertussis toxin (300 ng/mL) and then treated with S1P for 18 h. Bromodeoxyuridine (BrdU, 10 μM) was added to the medium for the last 40 min. The cells were fixed and incorporated BrdU was measured using an ELISA kit (Roche) according to the manufacturer's instructions.

### Telomerase activity assay

Telomerase activity was measured using a PCR-based telomeric repeat amplification protocol (TRAP) according to Kim et al. (1994). After treatment, cells were suspended in lysis buffer containing CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate). The cell lysate was centrifuged at 12 000 *g* for 20 min at 4°C and the protein concentration in supernatant was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Serially diluted cell extract (4–800 ng protein) was added to a reaction mixture containing TRAP buffer, 200 μM dNTP mixture, 10 mM 2-mercaptomethanol, 10 μM TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 10 μM CX primer (5′-CCCTTACCCTTACCCTTACCCTTA-3′), and AmpliTaq Gold DNA polymerase. The mixture was incubated at 25°C for 30 min followed by a 95°C incubation for 5 min. The mixture was then subjected to 31 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 40 s). The amount of amplified TRAP product was determined by quantifying SYBR Green fluorescence.

### Morphology assay

Cells were plated on 48-well plates at $4 \times 10^4$ cells/well and cultured for 2 days. Cells were pre-treated with U0126 (10 μM), Pertussis toxin (300 ng/mL) or Y-27632 (BioMol, 10 μM) for 3 h and then treated with S1P or LPA for 3 h. Cell morphology was photographed under phase-contrast microscopy.

## Results

### Agonist induced [$^{35}$S]GTPγS labeling in embryonic brain

In order to determine whether S1P activated GPCRs in early brain development, we performed [$^{35}$S]GTPγS autoradiographic studies in brain sections from E15, E16, E18 and E21 rat embryos. In E15 brains, 0.1 to 10 μM S1P dose-dependently increased [$^{35}$S]GTPγS labeling in neuroepithelial areas as well as differentiating fields (containing post-mitotic neurons) (Figs 1 and 2). The less potent S1P congener, dihydrosphingosine-1-phosphate (DH-S1P), did not significantly enhance [$^{35}$S]GTPγS binding at 0.1 μM (Fig. 1). However, the general pattern of [$^{35}$S]GTPγS labeling obtained with 0.1 and 10 μM DH-S1P was identical to that of S1P, although its intensity was lower (Fig. 1), suggesting that S1P- and DH-S1P-induced labeling was accounted for by S1P receptors. In contrast to S1P-induced labeling, 10 μM LPA enhanced binding was only observed in the cortical subventricular zone at E15 and E18 (Figs 2 and 3). At E18 and E21, S1P-induced [$^{35}$S]GTPγS binding had nearly disappeared in differentiating fields, and was mostly observed in neuroepithelial layers throughout the brain (Figs 3 and 4). At E21, LPA-induced [$^{35}$S]GTPγS was still observed in the cortical subventricular zone, but was also present in the glia limitans, hypothalamus, and in midbrain.

### Characterization of neural progenitor cells cultured from embryonic rat hippocampus

To examine the effects of S1P on cell signaling in neural progenitor cells, embryonic rat hippocampal cells were cultured in the presence of FGF-2. These cells showed

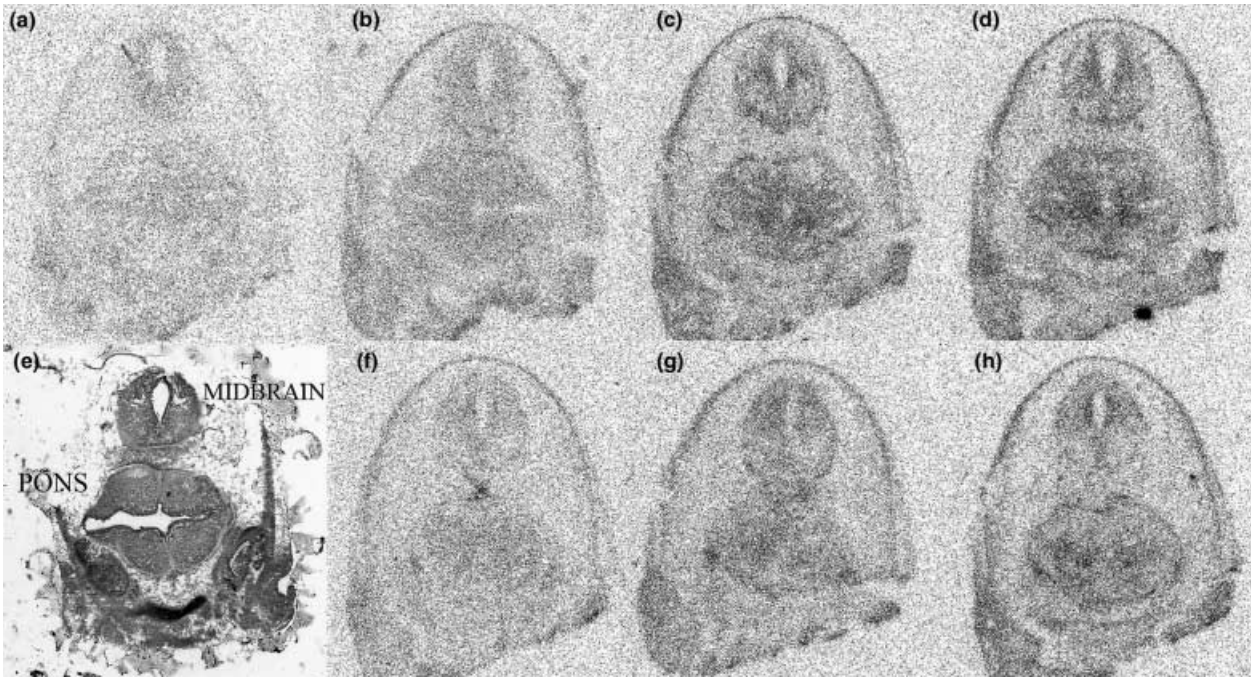


**Fig. 1** Distribution of [$^{35}$S]GTPγS labeling in frozen cryostat sections of an E15 rat embryo brain. Binding pattern is shown at the level of the brainstem in the absence of added agonist (a), in the presence of 0.1, 1 and 10 μM S1P (b, c, d), and in the presence of 0.1, 1 and 10 μM dihydro-S1P (f, g, h). (e) is a basic fuchsin histological staining of a cryostat section consecutive to those used for [$^{35}$S]GTPγS autoradiography. Note that both S1P and dihydro-S1P induce [$^{35}$S]GTPγS labeling in subventricular zones, but the former agonist does so at lower concentrations.

nestin immunoreactivity, a marker of neural stem/progenitor cells (Fig. 5a). In contrast, these cells were negative for a neuronal (TuJ1) or astrocytic marker (GFAP). The same cell preparation was able to form neurospheres on culture dishes without poly-lysine coating (data not shown).

Neural progenitor cells differentiated into neurons (TuJ1 positive, green in Fig. 5b), astrocytes (GFAP positive, red in Figs 5b and d) and oligodendrocytes (O4 positive, green in Fig. 5d) in differentiation medium lacking FGF-2 but containing 1% serum. High serum concentration (10% in Fig. 5c) induced cells to differentiate into astrocytes rather than neurons (compared with Fig. 5b). Differentiation of neural progenitor cells was confirmed by western blotting in which nestin was decreased and TuJ1 and GFAP increased after deprivation of FGF-2 and addition of 1% serum (Fig. 5e). Neural progenitor cells were allowed to differentiate in the presence or absence of S1P (1 μM) for 7 days. In the absence of serum, S1P enhanced neural differentiation as determined by decrease in nestin expression, increase in neural differentiation marker cyclin-dependent kinase inhibitor p27, neuronal marker MAP2 protein and glial marker GFAP (Fig. 12). These effects of S1P were not observed in the presence of 1% serum.

RT-PCR analysis was performed to determine which members of the S1P receptor family were expressed by hippocampal neural progenitor cells. As shown in Fig. 5(f), mRNA for $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ receptors was detected in growing neural progenitor cells in the presence of FGF-2. $S1P_2$, $S1P_3$, and $S1P_5$ receptor mRNA levels increased during differentiation. The expression pattern of these S1P receptor transcripts was different from that found in cortical neurons simultaneously prepared from E17 rat embryos, in which $S1P_2$ and $S1P_5$ mRNA was less abundant. We also examined the expression of GPR 3, 6, 12, 63, orphan GPCRs recently demonstrated to be responsive to S1P (Uhlenbrock *et al*. 2002; Niedernberg *et al*. 2003). The mRNA for these four receptors was detected in neural progenitor cells with different expression patterns (Fig. 5f).

### S1P induces activation of MAP kinase pathway in neural progenitor cells

MAP kinases play an important role in neural progenitor cell proliferation (Learish *et al*. 2000; Li *et al*. 2001) and S1P is known to activate these kinases in multiple systems (Sato *et al*. 1999; Pébay *et al*. 2001). We therefore examined the phosphorylation (activation) status of MAP kinases and ERK substrates in neural progenitor cells after S1P treatment (Fig. 6a). Phosphorylated ERK was markedly increased at 10 min by adding S1P in a concentration-dependent manner, while the total ERK level was unchanged. The increase of ERK phosphorylation was almost completely prevented by 10 μM U0126, an inhibitor of MEK (ERK activating kinase).

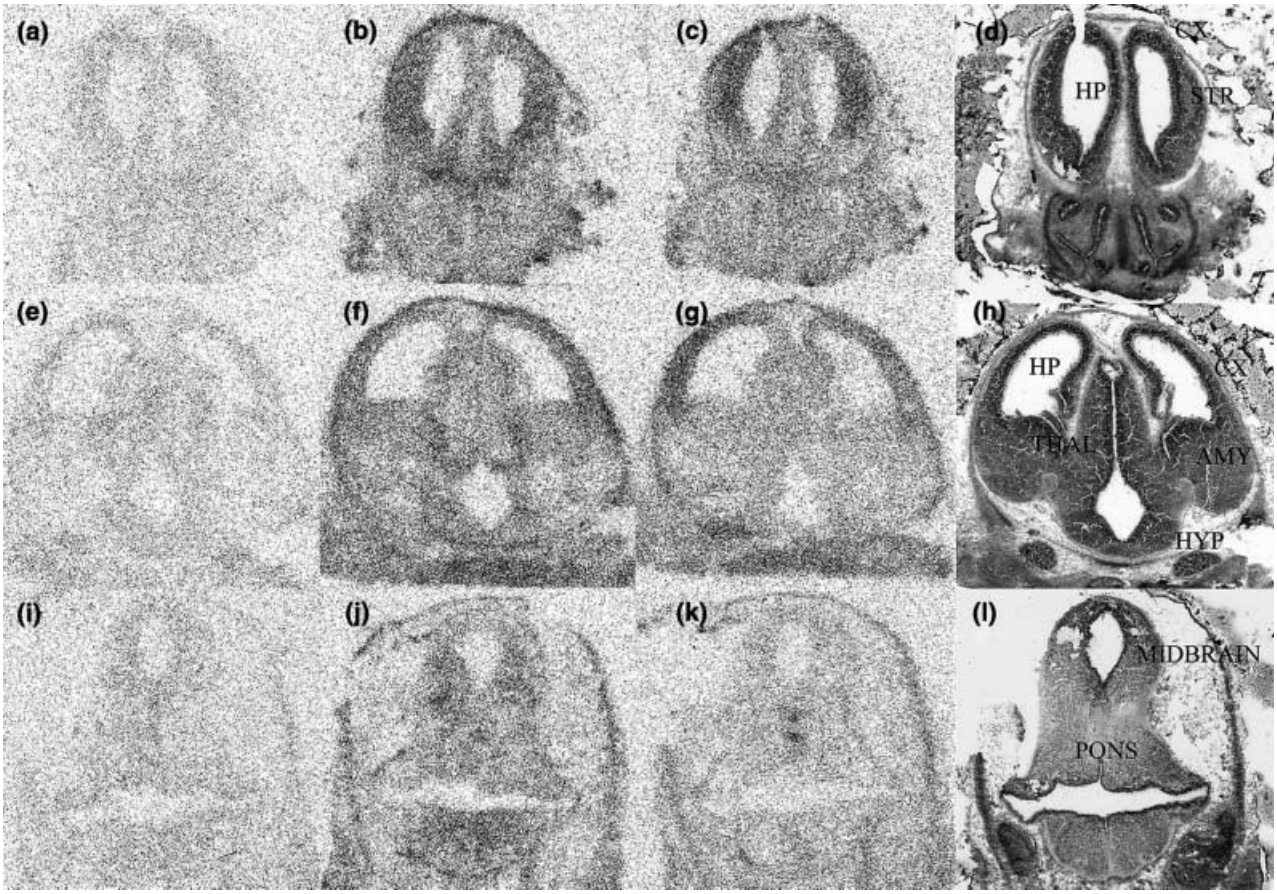


**Fig. 2** Distribution of [$^{35}$S]GTPγS labeling in frozen cryostat sections at different levels of an E15 rat brain. (a, e, i) Basal [$^{35}$S]GTPγS labeling. (b, f, j) and (c, g, k) [$^{35}$S]GTPγS labeling stimulated by 10 μM S1P and 10 μM LPA, respectively. (d, h, l) Basic fuchsin histological staining of cryostat sections consecutive to those used for [$^{35}$S]GTPγS autoradiography. AMY, amygdala; CX, neocortex; HP, hippocampus; HYP, hypothalamus; STR, striatum; THAL, thalamus.

MEK phosphorylation was also increased by S1P. p38 MAP kinase, another member of the MAP kinase family, was phosphorylated by S1P, but at relatively higher concentrations. p38 phosphorylation was partially prevented by U0126. Furthermore, we examined phosphorylation of p90 RSK and CREB, substrates of ERK pathway. Phosphorylation of these substrates were increased by S1P in a U0126-sensitive manner (Figs 6a–d). PD98059, another inhibitor of MEK, prevented S1P-induced phosphorylation of ERK (Fig. 7a). Phosphorylation of p38 was prevented only by higher concentration of U0126 (10 μM) and not by PD98059. S1P-induced phosphorylation of ERK was inhibited by pretreatment with Pertussis toxin (Fig. 7b), but not by Y-27632, an inhibitor of Rho-dependent kinase p160 ROCK (Fig. 7c). LPA did not induce ERK phosphorylation 10 min after treatment (Fig. 7d).

S1P induces expression of Egr-1 and FGF-2 through the ERK signaling pathway in cultured astrocytes (Sato *et al*. 1999). We therefore examined the expression of Egr-1 and FGF-2 in neural progenitors (Fig. 8). S1P treatment for 3 or 6 h increased mRNA levels of Egr-1 and FGF-2 as detected by RT-PCR (Fig. 8a). Furthermore, Egr-1 protein was also induced immediately after S1P treatment and the effect of S1P was completely prevented by U0126 (Fig. 8b).

### S1P induces proliferation and telomerase activation in neural progenitor cells

To examine the mitogenic effects of S1P, we cultured neural progenitors in the presence of FGF-2. FGF-2 was then withdrawn and cells were cultured for 18 h in the absence or presence of S1P. Cellular DNA synthesis was determined by measuring BrdU uptake. As shown in Fig. 9(a), S1P augmented BrdU uptake in the absence of FGF-2. LPA (1 μM), examined in parallel cultures, was ineffective (74 ± 14% of control). Basal as well as S1P-stimulated BrdU uptake was inhibited by U0126 (Fig. 9b). Pertussis toxin did not affect basal BrdU uptake, but almost completely prevented S1P-stimulated BrdU uptake (Fig. 9c).

It has been reported that cultured neural progenitor cells possess telomerase activity (Haik *et al*. 2000). To examine a possible effect of S1P on telomerase activity, we made serial

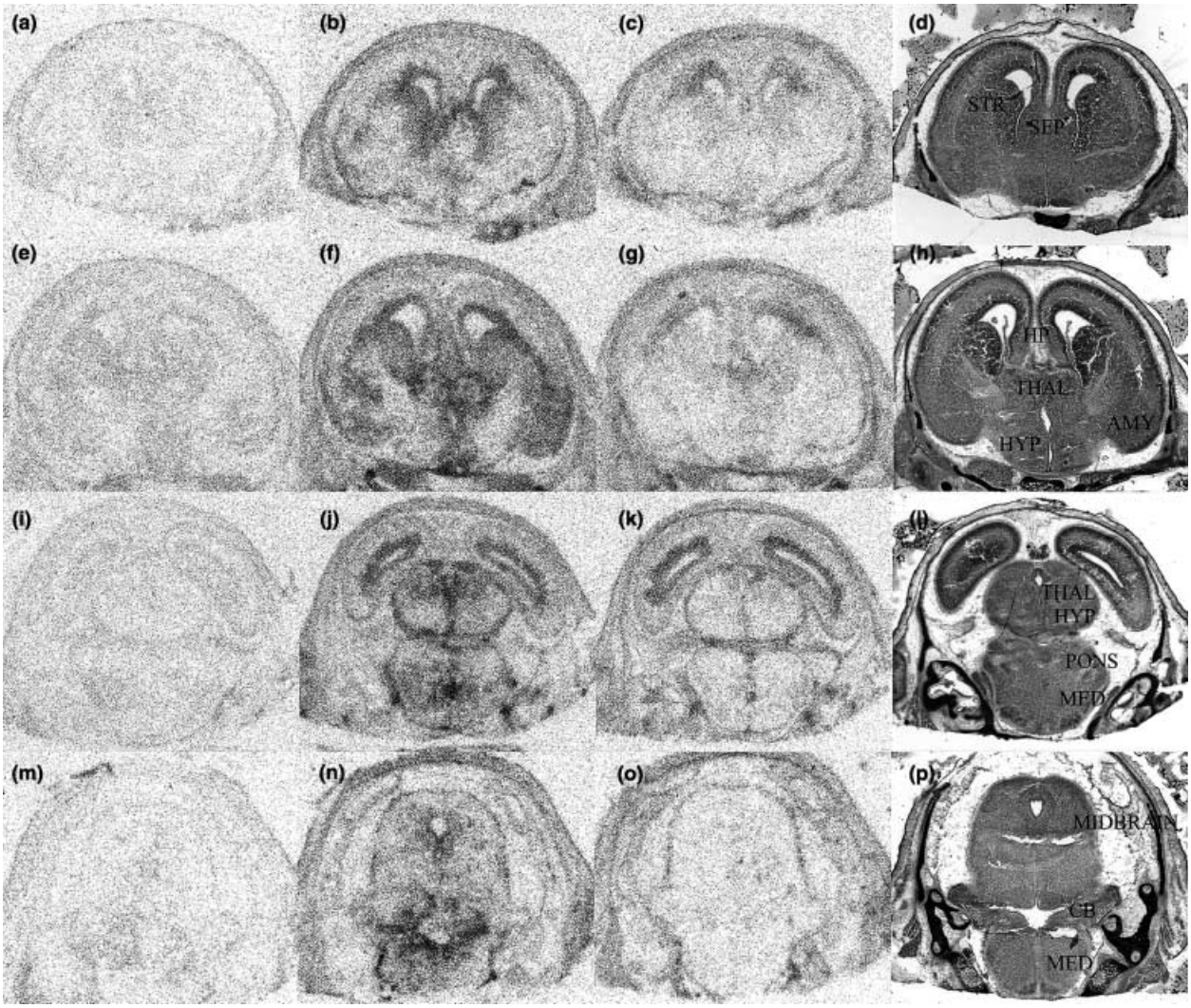


**Fig. 3** Distribution of [$^{35}$S]GTPγS labeling in frozen cryostat sections at different levels of an E18 rat brain. (a, e, i, m) Basal [$^{35}$S]GTPγS labeling. (b, f, j, n) and (c, g, k, o) [$^{35}$S]GTPγS labeling stimulated by 10 μM S1P and 10 μM LPA, respectively. (d, h, l, p) Basic fuchsin histological staining of cryostat sections consecutive to those used for [$^{35}$S]GTPγS autoradiography. AMY, amygdala; CB, cerebellum; HP, hippocampus; HYP, hypothalamus; MED, medulla; SEP, septum; STR, striatum; THAL, thalamus.

dilutions (5–600 ng) of progenitor cell extracts and measured telomerase activity by TRAP assay. The telomerase activity detected in neural progenitor cell extracts was similar to that detected in the HEK293 cell extracts used as positive controls (Kim *et al*. 1994) (Fig. 10a). In the absence of FGF-2, S1P (1–3 μM) increased telomerase activity about threefold, a stimulation comparable with that observed with FGF-2 (Fig. 10b). Both basal and S1P-stimulated telomerase activities were prevented by U0126 (Fig. 10c).

### S1P induces morphological changes of neural progenitor cells

After treatment with S1P (3 μM) or LPA (1 μM), neural progenitor cells became aggregated (Fig. 11a). This morphological change was transient, reaching a maximum 2–3 h after treatment and returning to baseline 18 h after treatment. Lactate dehydrogenase activity in culture medium or medium supernatant was not increased by S1P treatment for 24 h and 7 days, suggesting that S1P did not induce cell death or cell detachment from the culture dishes (data not shown). S1P-induced morphological changes were almost completely prevented by Y-27632 (Fig. 11d), but not by U0126 (Fig. 11b) or Pertussis toxin (Fig. 11c). A similar sensitivity to pharmacological inhibitors was observed for the response to LPA (data not shown).

## Discussion

Our [$^{35}$S]GTPγS autoradiography results suggest that brain areas with proliferating cells contain S1P receptors able to

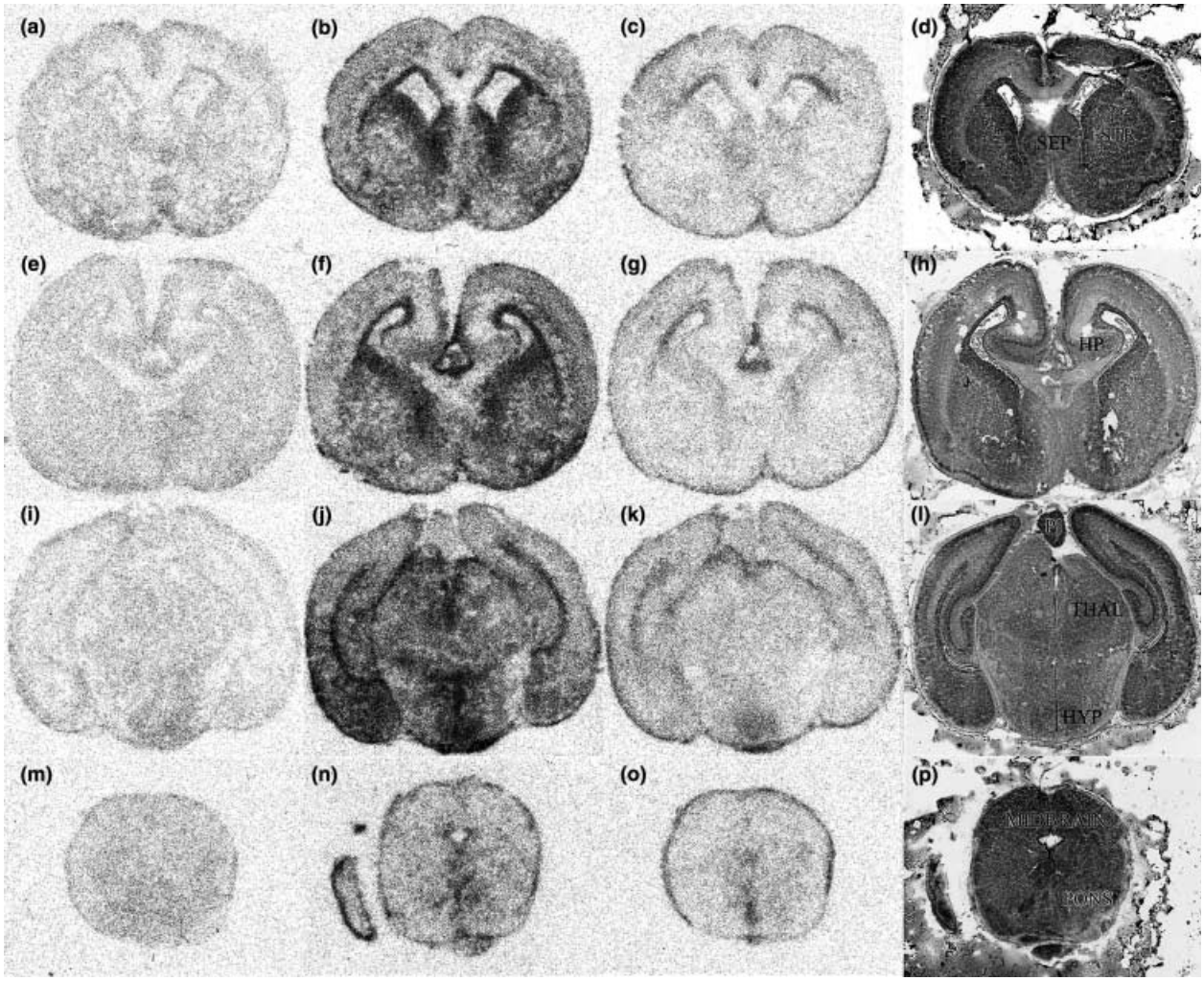


**Fig. 4** Distribution of [$^{35}$S]GTPγS labeling in frozen cryostat sections at different levels of an E21 rat brain. (a, e, i, m) Basal [$^{35}$S]GTPγS labeling. (b, f, j, n) and (c, g, k, o) [$^{35}$S]GTPγS labeling stimulated by 10 μM S1P and 10 μM LPA, respectively. (d, h, l, p) Basic fuchsin histological staining of cryostat sections consecutive to those used for [$^{35}$S]GTPγS autoradiography. HP, hippocampus; HYP, hypothalamus; MED, medulla; PI, pineal gland; SEP, septum; STR, striatum; THAL, thalamus.

activate G-proteins as early as E15. Using neural progenitor cells prepared from embryonic rat hippocampus, we show that S1P activates ERK via high-affinity Pertussis toxin-sensitive receptors, an effect that leads to BrdU incorporation and presumably cell proliferation. In addition, S1P induces morphological changes in these neural progenitor cell cultures, via a distinct, Pertussis toxin-insensitive, ERK-independent signal transduction pathway involving Rho kinases.

The S1P concentrations used in this study are comparable with the submicromolar levels found in serum (Yatomi *et al*. 1997). Recently, Palmer *et al*. (2000) demonstrated that neurogenesis in adult hippocampal subgranular zone occurs within an angiogenic niche. The proliferation clusters that generate neurons contain endothelial precursors and reside around small capillaries. In this context, it is interesting to note that S1P is released from platelets and activates endothelial cell proliferation (Yatomi *et al*. 2001) and therefore may play a role in the formation of proliferation clusters generating new neurons. It is also possible that S1P is synthesized and released by neural progenitors in an autocrine manner, because these cells express both S1P producing enzymes, sphingosine kinase 1 and 2 (RT-PCR data not shown). Although the effect of S1P on proliferation is weaker compared with that of FGF-2 (397 ± 104% of control in the present study), it should be noted that the FGF-2 concentration that typically induces neural stem/progenitor cell proliferation (0.3–3 ng/mL) is unlikely to be found under physiological conditions (5.9 pg/mL in normal human serum, Ii *et al*. 1993). Our recent analysis in adult hippocampus demonstrated that neurogenesis after brain injury was significantly decreased in FGF-2 deficient mice while

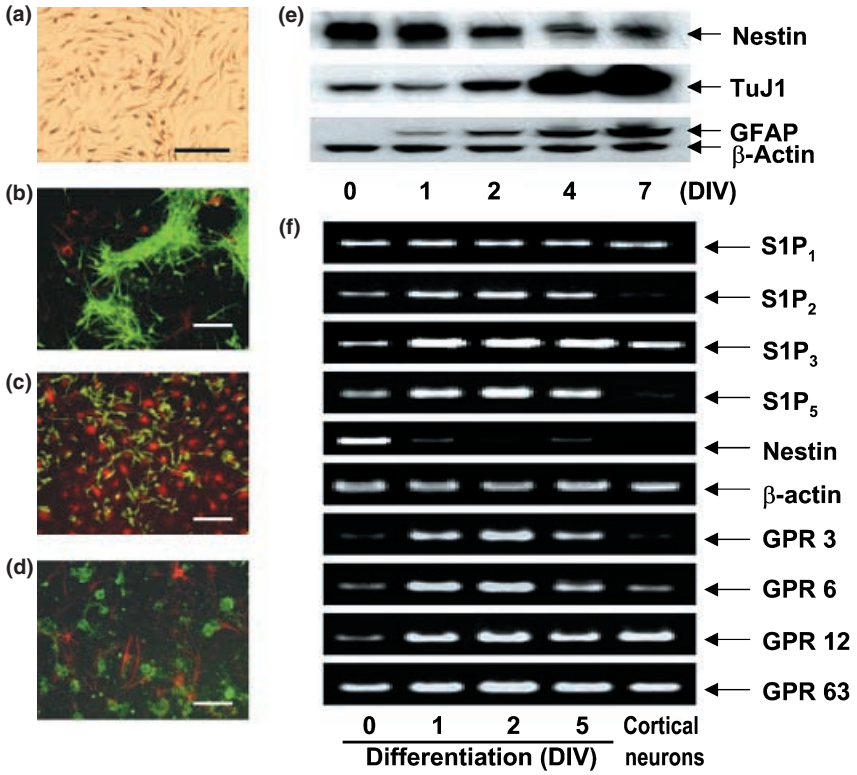


**Fig. 5** Characterization of neural progenitor cells cultured from embryonic rat hippocampus. Neural progenitor cells are prepared from E17 rat hippocampus and expanded in the presence of FGF-2. (a) Neural progenitor cells grown in the presence of FGF-2 were fixed and immuno-stained with antinestin antibody. (b–d) Neuronal progenitor cells were allowed to differentiate by removal of FGF-2 and addition of 1% (b, d) or 10% (c) fetal bovine serum for 7 days. The cells were immuno-stained with TuJ1 antibody, an early neuronal marker (green, b, c), anti-GFAP, an astrocyte marker (red, b, c, d) and anti-O4, an oligodendrocyte marker (green, d), scale bar = 50 μm. (e, f) Neural progenitor cells were allowed to differentiate by removal of FGF-2 and addition of 1% serum for the indicated days. In (e), expression of nestin, TuJ1 and GFAP were determined by western blotting. The results are from the same blot and expression of β-actin is shown to confirm uniform loading. In (f), RT-PCR analysis of S1P receptor family transcripts, nestin, β-actin and some orphan G-protein-coupled receptors (GPRs) were performed using total RNA prepared from neural progenitor cells and cortical neurons, both obtained from E17 rat embryos.

basal levels of neurogenesis were unaffected (Yoshimura *et al*. 2001). Thus, FGF-2 probably plays role on neurogenesis under specific circumstances (i.e. after injury).

Our results suggest that proliferation of neural progenitor cells follows S1P-mediated activation of the ERK pathway via Pertussis toxin-sensitive GTP-binding proteins such as $G_i$. This finding is in agreement with the fact that several subtypes of Pertussis toxin-sensitive GPCRs mediate cellular responses to S1P (Lee *et al*. 1998; Lee *et al*. 1999; Wang *et al*. 1999). Most effects of S1P on progenitor cells were mediated by ERK, as shown by their sensitivity to U0126, a potent inhibitor of the ERK activating kinase MEK (Favata *et al*. 1998). U0126 also prevented phosphorylation of ERK and substrates of the ERK pathway, p90 RSK and CREB (Fig. 6). Relatively higher S1P concentrations also resulted in p38 MAP kinase phosphorylation (Fig. 6), another member of the MAP kinase family. S1P-induced p38 phosphorylation was partially prevented by U0126, in agreement with the known lower affinity of this inhibitor for p38 (Favata *et al*. 1998; Davies *et al*. 2000), but was insensitive to the MEK inhibitor PD98059 (Fig. 7a). Phosphorylation of ERK but not p90 RSK and CREB in the absence of S1P was prevented by U0126 (Fig. 6), suggesting the existence of alternative, ERK-independent effects on these kinases.

The pathway linking ERK activation by S1P and cellular proliferation is not well defined. In cultured astrocytes, S1P induces expression of FGF-2 and Egr-1 through ERK (Sato *et al*. 1999). We found that S1P increased expression of FGF-2 and Egr-1 in neural progenitors after FGF-2 deprivation (Fig. 8). FGF-2 is known to stimulate Egr-1 expression in endothelial cells (Santiago *et al*. 1999) and Egr-1 can in turn activate FGF-2 transcription (Biesiada *et al*. 1996; Wang *et al*. 1997). Recently, Fahmy *et al*. (2003) demonstrated that Egr-1 supports FGF-2-dependent angiogenesis, especially endothelial cell proliferation. It is noteworthy that several GPCRs activate ERK pathway via transactivation of growth factor receptor tyrosine kinases. In

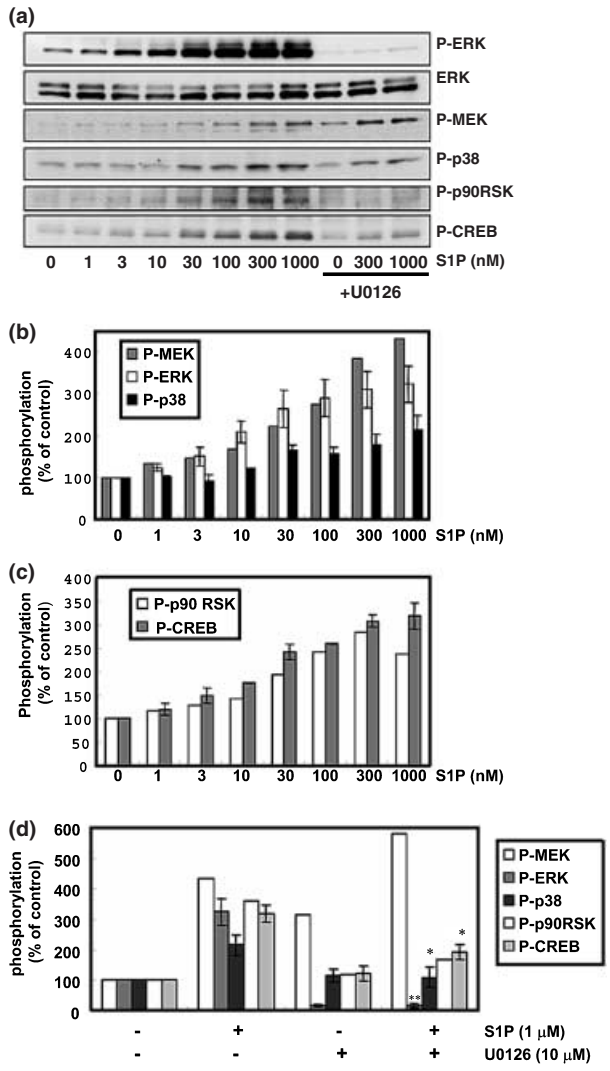


**Fig. 6** S1P induces activation of MAP kinase pathway in neural progenitor cells. Neural progenitor cells were starved of FGF-2 and treated with the indicated concentrations of S1P for 10 min. Protein samples were analyzed by western blotting using antibodies against the phosphorylated forms of MAP kinases and substrates of ERK. (a) Representative autoradiograms. From the top, levels of phosphorylated ERK, total ERK, phosphorylated MEK, phosphorylated p38, phosphorylated p90RSK, and phosphorylated CREB were determined. In some samples, U0126, an inhibitor of MEK, was added at 10 μM prior to S1P treatment. (b–d) The amounts of phosphoproteins are expressed as percentage of those measured in vehicle-treated sample. Data represent mean ± SEM of three independent experiments (phosphorylated ERK, phosphorylated p38 and phosphorylated CREB) or mean of two independent experiments (phosphorylated MEK and phosphorylated p90RSK). *$p < 0.05$ and **$p < 0.01$ between S1P-treated groups and S1P plus U0126-treated groups by Student's *t*-test.

particular, S1P stimulation activates PDGF receptors in smooth muscle cells (Waters *et al*. 2003) and VEGF receptor in endothelial cells (Tanimoto *et al*. 2002). In turn, PDGF

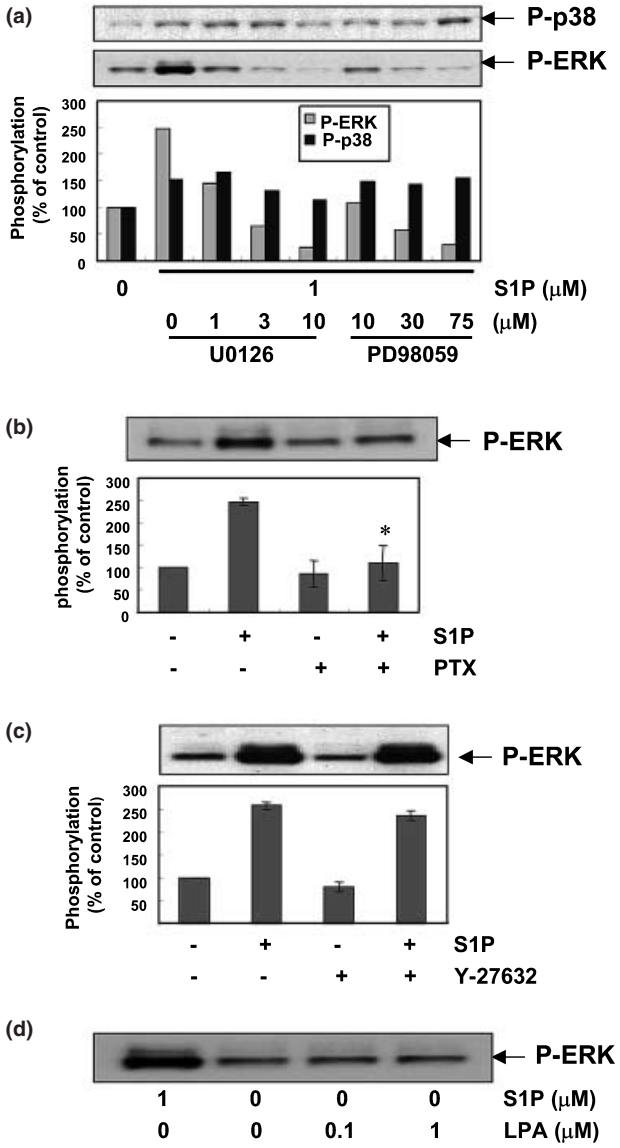


**Fig. 7** Characterization of S1P-induced activation of MAP kinase in neural progenitor cells. Neural progenitor cells were starved of FGF-2 and treated with 1 μM S1P for 10 min. During FGF-2 withdrawal, various inhibitors were added prior to S1P treatment. Protein samples were analyzed by western blotting using antibodies against the phosphorylated forms of MAP kinases. (a–c) Cells were pre-treated with or without (a) indicated concentrations of MEK inhibitors U0126 or PD98059, (b) Pertussis toxin (PTX), 300 ng/mL and (c) Y-27632, 10 μM, and then treated with 1 μM S1P for 10 min. Upper panels, representative autoradiograms. Lower panels, amounts of phosphoproteins expressed as percentage of vehicle-treated samples. Data are mean of two independent experiments (a) or mean ± SEM of three independent experiments (b, c). *$p < 0.05$ between S1P-treated groups and S1P plus PTX-treated groups by Student's *t*-test. (d) Cells were treated with the indicated concentrations of S1P or lysophosphatidic acid (LPA) for 10 min. Data are from a representative experiment that was repeated twice with similar results.

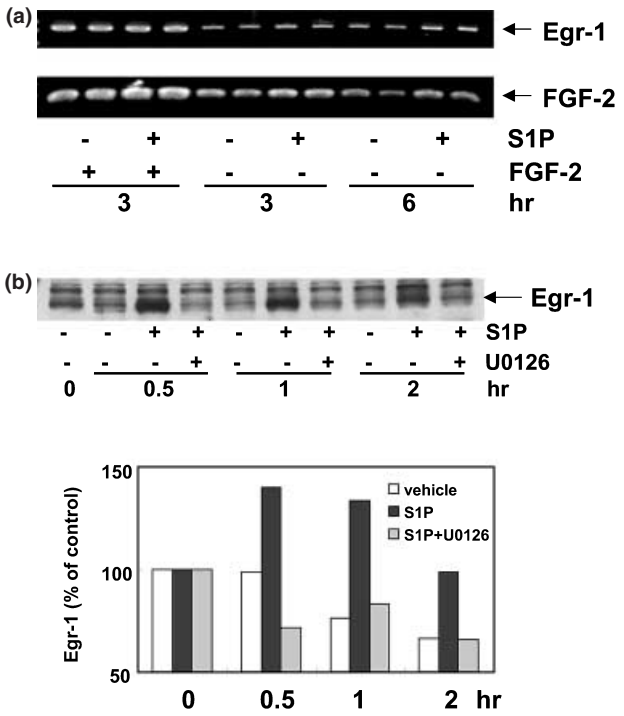


**Fig. 8** Induction of Egr-1 and FGF-2 by S1P treatment. Neural progenitor cells were deprived of FGF-2 and treated with 1 μM S1P for the indicated times. During FGF-2 withdrawal, cells were pre-treated with or without 10 μM U0126. (a) RT-PCR analysis of Egr-1 and FGF-2 transcripts. Data are representative of two independent experiments. (b) Western blots obtained using an antibody against Egr-1 (note that the upper protein band is non-specific). Lower panels: amounts of proteins expressed as percentage of non-treated samples. Data are mean of two independent experiments.

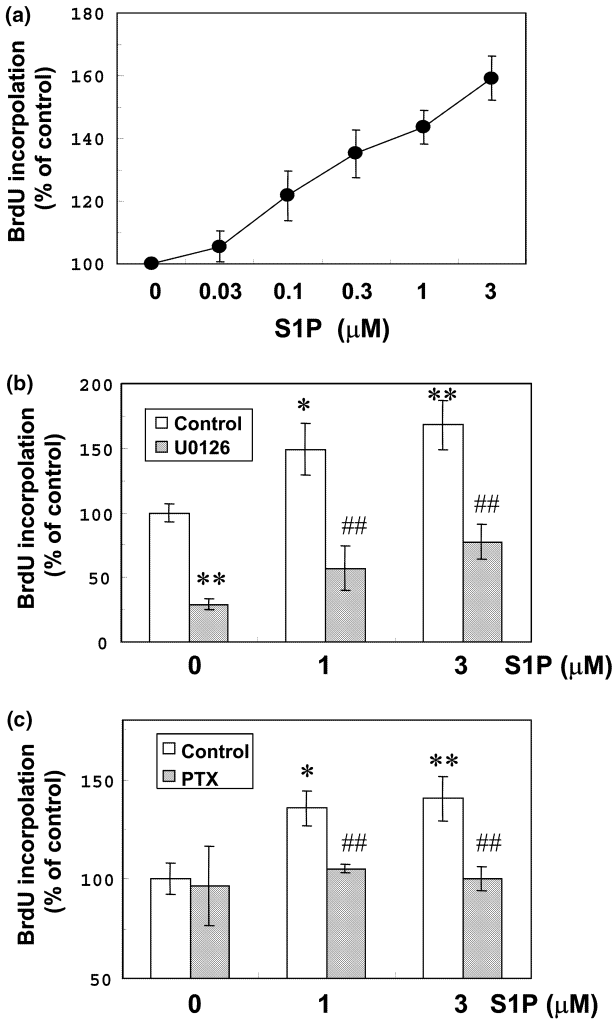


**Fig. 9** S1P induces proliferation of neural progenitor cells. (a) Neural progenitor cells were starved of FGF-2 and treated with the indicated concentrations of S1P for 18 h. Proliferating cells were then labeled with BrdU (10 μM) and the amount of incorporated BrdU was measured by ELISA. In simultaneous determination on sister cultures, BrdU incorporation in the presence of FGF-2 (10 ng/mL) or LPA (1 μM) were 397 ± 104 or 74 ± 14% of control, respectively. Data represent mean ± SEM from four independent experiments. (b, c) Neural progenitor cells were starved of FGF-2 and pre-treated for 3 h with (b) U-126 (10 μM) or (c) Pertussis toxin (300 ng/mL). Thereafter, cells were treated with (1 μM, 3 μM) or without S1P for 18 h. Incorporated BrdU was measured by ELISA. Data are mean ± SEM ($n = 3$) from a representative experiment that was repeated twice with similar results. *$p < 0.05$ and **$p < 0.01$ compared with vehicle-treated samples and ##$p < 0.01$ between S1P-treated groups and S1P plus U0126 (b) or PTX (c)-treated groups by Student's *t*-test.

stimulation can lead to S1P receptor activation via sphingosine kinase activation (Hobson *et al.* 2001). It is therefore conceivable that a cross-talk exists between S1P and FGF-2 signaling in neural progenitor cells, although we did not directly investigate this hypothesis.

In mitotic somatic cells, telomeres shorten during each cell division and may act as a mitotic clock to eventually signal cell cycle exit and cellular senescence (Liu 1999; Collins and Mitchell 2002). Telomerase prevents telomere shortening and has also been shown to be involved in cell proliferation and anti-apoptotic activity (Fu *et al.* 1999; Klapper *et al.* 2001; Mattson and Klapper 2001; Oh *et al.* 2001; Inui *et al.* 2002; Smith *et al.* 2003). It is present in human germ line cells and cancer cells, but absent in most normal adult somatic tissues. Telomerase activity is detected in developing brain but not in adult brain (Klapper *et al.* 2001). Recently, high FGF-2 regulated telomerase activity has been demonstrated in mouse cortical neural progenitor cells (Haik *et al.* 2000). We observed similar findings in hippocampal neural progenitor cells (Fig. 10). Interestingly, S1P also induced activation of telomerase activity (Fig. 10b). In regenerating hepatocytes, growth factor-induced telomerase activation is prevented by MEK inhibitor (Inui *et al.* 2002), suggesting the role of ERK in telomerase activation. Indeed, both basal and S1P-induced telomerase activities were prevented by U0126 (Fig. 10c). To our knowledge, telomerase activation by a small GPCR ligand

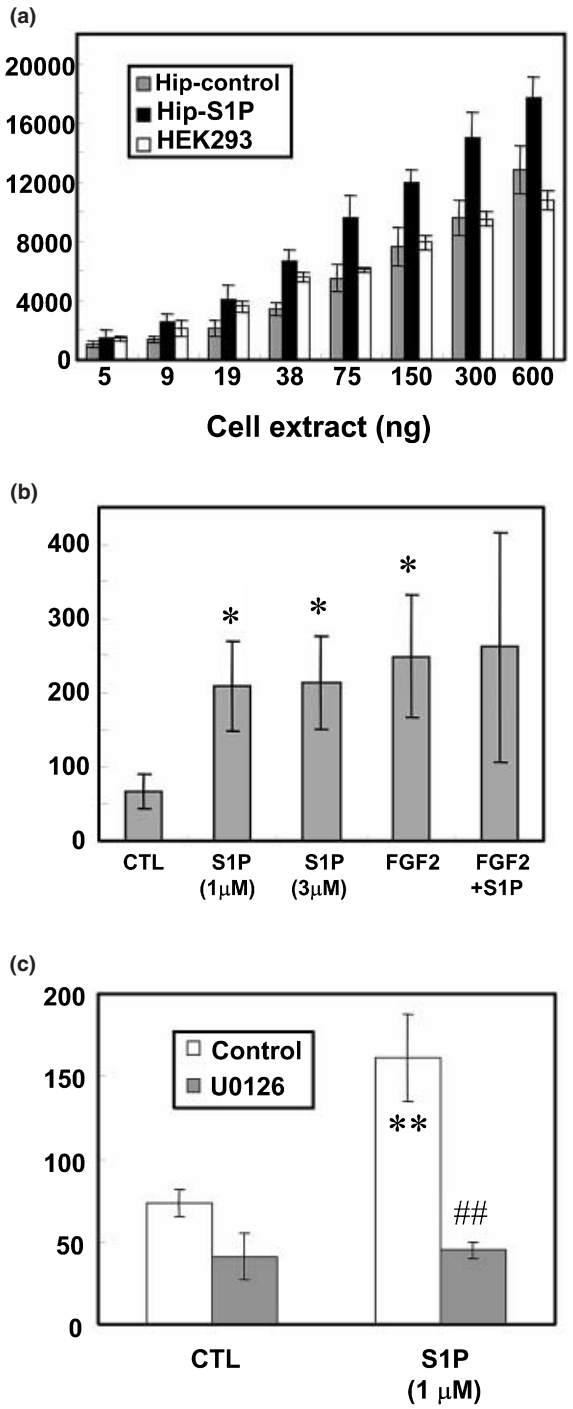


**Fig. 10** S1P enhanced telomerase activity in neural progenitor cells. Neural progenitor cells were starved of FGF-2 and treated without or with S1P (1 μM, 3 μM) for 18 h. The cellular extracts were serially diluted and telomerase activities in each sample were analyzed by TRAP assay. Telomerase activity in HEK293 cells was measured simultaneously. (a) Amplified telomeric repeats were stained with SYBR Green and fluorescence units are presented. (b) Telomerase activities in neural progenitors after respective treatments (150 ng each tube) are calculated as equivalents of activity in HEK293 cell extract (ng). (c) Neural progenitor cells were pre-treated for 3 h with U0126 (10 μM, filled bar). Thereafter, cells were treated as in (b). Data are mean ± SEM ($n = 3$) from a representative experiment that was repeated twice with similar results. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with vehicle-treated samples and $\#\#p < 0.01$ between S1P-treated groups and S1P plus U0126-treated groups by Student's *t*-test.

has not been documented previously. These data suggest that regulation by S1P of telomerase activity can be a determinant for the brain development.

S1P also induced transient morphological changes on neural progenitor cells (Fig. 11), but via a signal transduction pathway distinct from that mediating ERK activation and proliferation. Y-27632, an inhibitor of Rho-dependent kinase p160 ROCK (Hirose *et al*. 1998), but not U0126 or Pertussis toxin, prevented the effects of S1P on cell morphology (Figs 11b–d). However, Y-27632 had no effect on S1P-induced ERK phosphorylation (Fig. 7c). Thus, S1P-induced morphological changes are mediated by Rho, and possibly $G_{13}$ (Lee *et al*. 1999), but not by Pertussis toxin-sensitive G-proteins-ERK pathway.

In addition to the effect on proliferation and morphology, S1P enhanced neural differentiation in the absence of serum (Fig. 12). S1P enhanced p27 expression, a cyclin-dependent kinase inhibitor induced during neuronal differentiation (Perez-Juste and Aranda 1999; Sasaki *et al*. 2000). One percent serum enhanced neural differentiation (Figs 5 and 12). Because serum contains S1P, it is possible that part of the effect of serum on neuronal differentiation is mediated by S1P and that the effect of S1P was masked by the presence of serum.

LPA, a lipid related to S1P, had no effect on ERK phosphorylation (Fig. 7d) and proliferation of neural progenitor cells. However, LPA was more potent than S1P at inducing morphological changes (Fig. 11a), using a similar signal transduction pathway. $LPA_1$ receptors are expressed abundantly in the ventricular zone of the developing cerebral cortex (Hecht *et al*. 1996). These receptors might be involved in cortical development and play a role in the rounding-up phase of interkinetic nuclear migration of cortical neuroblasts (Fukushima *et al*. 2000). Furthermore, LPA induces morphological changes and migration of early post-mitotic neurons, effects that are mediated by Rho proteins (Fukushima *et al*. 2002). The results of the present study raise the possibility that S1P might similarly influence interkinetic nuclear migration as well as the proliferation of neural progenitor cells.

The identity of the receptor subtypes that mediate the effects of S1P and LPA in the present study is not known. In hippocampal neural progenitor cells, mRNAs encoding $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$ receptor transcripts were detected by RT-PCR (Fig. 5f). $S1P_2$, $S1P_3$, and $S1P_5$, but not $S1P_1$, receptor mRNA levels increased during differentiation (Fig. 5f). The presence of S1P (1 μM) in the culture medium did not affect expression of these transcripts during

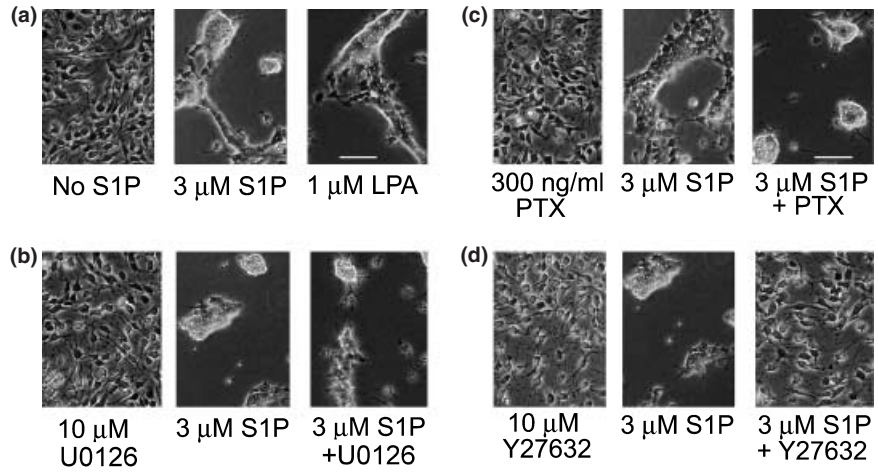


**Fig. 11** S1P induces morphological changes of neural progenitor cells. (a) Neural progenitor cells were treated with S1P (3 μM) or LPA (1 μM) for 3 h, and their morphology was photographed under phase-contrast microscope. (b–d) Drugs were added 3 h before S1P treatment (b, 10 μM U0126; c, 300 ng/mL Pertussis toxin; d, 10 μM Y-27632). S1P and LPA induced progenitor cells aggregation within 3 h of treatment. Normal morphology was observed 24 h after treatment with either lipid (not shown). Bar = 50 μm. Data are representative of three independent experiments that gave similar results.

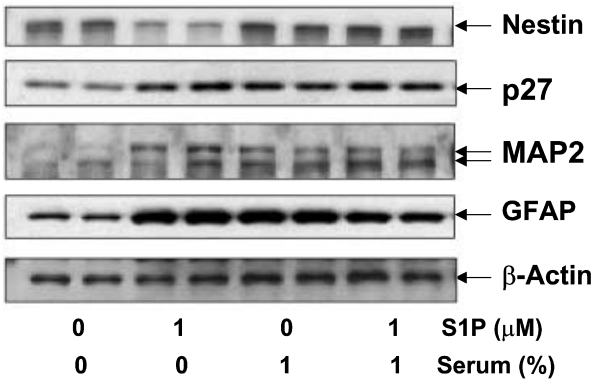


**Fig. 12** S1P enhances differentiation of neural progenitor cells. Neuronal progenitor cells were allowed to differentiate by removal of FGF-2 in the presence or absence of 1 μM S1P and 1% fetal bovine serum for 7 days. Expression of nestin, p27, MAP2, GFAP and β-actin were determined by western blotting. Data are representative of two independent experiments that gave similar results.

differentiation (data not shown). Of the different S1P receptor subtypes, $S1P_1$ and $S1P_5$ are preferentially coupled to Pertussis toxin-sensitive G proteins such as $G_i$. $S1P_2$ and $S1P_3$, but not $S1P_1$, activate $G_q$ and $G_{13}$ (Windh *et al*. 1999). We have previously shown that only receptors coupled to $G_{i/o}$ can be mapped using [$^{35}$S]GTPγS autoradiography (Waeber and Moskowitz 1997). It is therefore tempting to speculate that the S1P receptors accounting for the proliferative effects of S1P are either $S1P_1$ or $S1P_5$. *In situ* hybridization studies show that the pattern of S1P-induced [$^{35}$S]GTPγS labeling in rat embryo brain is virtually superimposable with that of $S1P_1$ mRNA distribution in mouse embryo brain (McGiffert *et al*. 2002). In addition, $S1P_1$ mRNA was found in cells incorporating BrdU in E14 mouse embryo brains including hippocampus (McGiffert *et al*. 2002), strongly suggesting that this subtype may be critical during brain development and mediates the proliferative effects of S1P. Recently, knockout mice for several S1P receptor genes have been reported. Deficiency in $S1P_1$ receptor resulted in prenatal death between E12.5 and E14.5 owing to massive hemorrhage (Liu *et al*. 2000), pointing to a crucial role of this receptor during vascular development, but masking a potential role of this receptor in brain ontogeny. In the present study, morphological changes were observed at higher S1P concentrations (> 0.1 μM) than the effect on ERK activation and proliferation. The insensitivity of this response to Pertussis toxin suggests that the pleiotropic effects of S1P might be mediated by a single receptor coupled to various downstream effector systems with different efficiency, or by different S1P receptor subtypes, such as $S1P_2$ or $S1P_3$ subtypes. Interestingly, while $S1P_2$ knockout mice develop normally, they show increased excitability of neocortical pyramidal neurons consistent with a role of $S1P_2$ receptors in brain development (MacLennan *et al*. 2001). Finally, a possible role of orphan GPCRs recently demonstrated to be responsive for S1P (Uhlenbrock *et al*. 2002; Niedernberg *et al*. 2003) remains to be elucidated.

In conclusion, these findings indicate that S1P plays an important role during brain development, probably by acting via different receptor subtypes. Selective receptor agonist or antagonist, as well as tissue-specific receptor knockout, will be useful to determine the precise function of each these subtypes in embryonic and adult brain.

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