[^{3}H]Thymidine Incorporation Assay

Cortical embryonic NPCs were seeded in 96-well plates (1×10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C with b-FGF (5 ng/mL). Vehicle or peptide (PACAP38, 0–100 nM; maxadilan, 0–10 nM; or M65, 10 nM) was then added; after 1 h, 1 μCi/well [^{3}H]thymidine was also added. After a 7-h incubation, the cells were washed extensively with medium, and [^{3}H]thymidine incorporation was measured using a β-counter.

For the CM proliferation assay, embryonic cortical NPCs were seeded in 96-well plates (1×10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C. Medium was then replaced with NPC conditioned medium or control medium. [^{3}H]thymidine incorporation and measurement were performed as described above.

To assess PAC1 signaling, cortical embryonic NPCs were seeded in 96-well plates (10^4 cells/well) in 100 μL of medium and cultured for 24 h at 37°C. Cells were pre-incubated for an additional 1 h with vehicle and signal transduction inhibitors (H89, 10 μM 2-APB, 20 μM; chelerythrine, 50 μM), and then maxadilan (10 nM) was added. [^{3}H]thymidine incorporation was measured as described above.

Intracellular ATP Assays

It has been previously reported that intracellular ATP levels correlate with cell number (Crouch et al., 1993). Embryonic cortical NPCs were seeded in 48-well plates (4.5×10^4 cells/well) in 400 μL of medium and cultured for 24 h at 37°C with b-FGF (5 ng/mL). After 24 h, each peptide (PACAP38, 0–100 nM; maxadilan, 0–10 nM; PACAP(6–38), 10 nM) was added to these NPC cultures. After a 6 or 24-h incubation with the peptides, intracellular ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Radioimmunoassay for PACAP38

PACAP38 concentration was measured in conditioned medium derived from NPCs using a radioimmunoassay (RIA). Cell-free conditioned medium (100 μL) and conditioned medium derived from NPCs (100 μL) were incubated with polyclonal anti-PACAP38, which was rehydrated in RIA buffer (Phoenix Pharmaceuticals, Belmont, CA) for 24 h at 4°C. These reactions were incubated with ^{125}I-PACAP(31–38) (50 cpm/μL) for an additional 24 h at 4°C. Second antibody reaction/separation and detection of ^{125}I in the pellets were performed in a scintillation well gamma counter according to the instrument manufacturer's instructions (Phoenix Pharmaceuticals, Belmont, CA).

Intracellular cAMP Measurement

Cortical embryonic NPCs were seeded in 96-well plates and treated with PACAP38 (100 nM) or maxadilan (10 nM) for 24 h. cAMP levels were assayed by the cAMP-Screen™ System (Applied Biosystems).

Intracellular Calcium Imaging

Cortical embryonic NPCs were seeded in 96-well plates and cultured for 24 h as described above for the intracellular cAMP measurement. The intracellular concentration of free calcium ([Ca^{2+}]i) in cultured NPCs was monitored using the Calcium Kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. For a negative control, cells were pre-incubated for an additional 1 h with BAPTA-AM (100 μM), and then PACAP38 (100 nM) or maxadilan (10 nM) was added.

RESULTS

Expression of PAC1 and PACAP in Embryonic Cortical NPCs In Vivo and In Vitro

To generate a highly purified population of embryonic cortical NPCs, we prepared a secondary culture of NPCs (Li et al., 2001; Nakashima et al., 1999). Most of these cells were positive for nestin, a specific marker for NPCs in developmental brain, but less than 0.5% were immunoreactive for β III tubulin and GFAP. Gal C positive cells were not found (data not shown).

We used RT-PCR and immunohistochemical methods to investigate PAC1 and PACAP expression in cultured mouse embryonic cortical NPCs. RT-PCR detected both PAC1 and PACAP mRNAs in the NPCs and mouse telencephalon at embryonic day 14.5 (Fig. 1A). Other PACAP receptors, VPAC1 and VPAC2, mRNAs were expressed at a much lower level [(0.01 ± 1.7)% and (0.11 ± 2.3)% (mean ± S.E.M.), respectively] than PAC1 mRNA (33.1 ± 0.23)% (Fig. 1A). In addition, the mRNA for another PAC1 ligand, VIP, was not detected in embryonic cortical NPCs (Fig. 1A). In immunohistochemistry experiments, PACAP protein was expressed in nestin-immunoreactive NPCs, and most nestin-immunoreactive NPCs expressing PAC1 colocalized with PACAP in the VZ and SVZ (Fig. 1B). We also detected PACAP and PAC1 in nestin-immunoreactive cultured embryonic cortical NPCs. Over 99.8% of the PAC1-expressing cells co-expressed PACAP (Fig. 1C).

Activation of PAC1 Signaling Induces Embryonic Cortical NPC Proliferation in the Presence of b-FGF in an Autocrine Manner

We performed a [^{3}H]thymidine incorporation assay and an ATP assay, which measures intracellular ATP

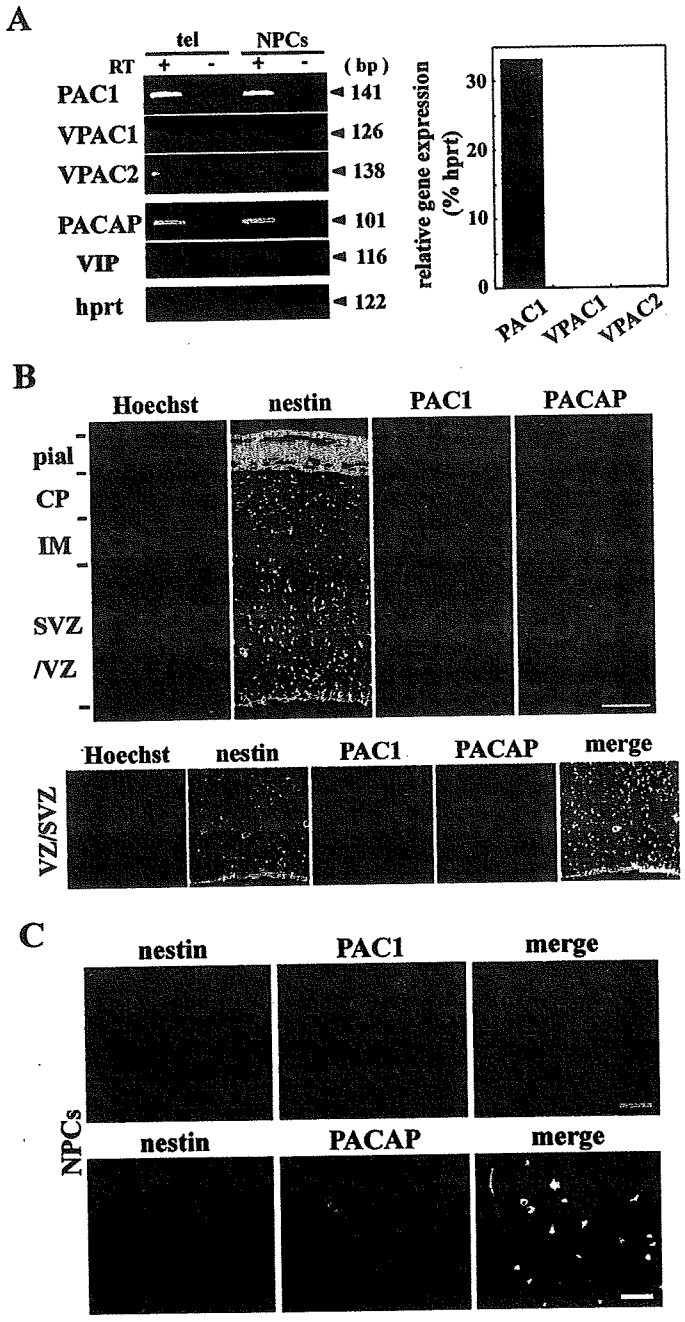

Fig. 1. NPCs express the PACAP receptors PAC1, VPAC1 and VPAC2, and their ligand, PACAP. **A:** RT-PCR was performed as described in Material and Methods. PCR products corresponding to these receptor and ligand genes were loaded onto a 3% agarose gel, as indicated at the left. The size of the PCR products is indicated on the right. Quantitative analysis of mRNA transcripts for PACAP receptors in NPCs by SYBER green-based PCR 7700 system. Data represents expression level of individual PACAP receptors relative to the expression level of hprt. **B,C:** PACAP is an autocrine factor of NPCs. PAC1 and PACAP protein expression was determined by immunofluorescence in vivo (B) and in vitro (C). Nestin-immunoreactive cells (cyan) in E14.5 mouse embryonic telencephalon also expressed PAC1 (green) and PACAP (red). The lower panels are high magnifications of the upper panels (B). Cell nuclei were counterstained with Hoechst 33258 (blue, B). Almost all nestin-immunoreactive NPCs (red, C) expressed PAC1 (green, top panel in C) and PACAP (green, lower panel in C). Cell nuclei were counterstained with Hoechst 33258 (blue, C). Scale bars: (B, C) 50 μm.

levels and hence indicates the viable cell number, to determine whether PAC1 signaling promotes NPC proliferation. A 6-h incubation neither with PACAP38 (0–100 nM), the natural ligand for PAC1, nor with maxadilan (0–1 nM), a specific agonist for PAC1, induced DNA synthesis in the absence of b-FGF (Fig. 2A). In contrast, both reagents increased [^{3}H]thymidine incorporation in a dose-dependent manner in the presence of b-FGF without altering the viable cell number as assessed by the ATP assay (Figs. 2A,B). After incubation for 24 h, maxadilan increased intracellular ATP levels in the presence of b-FGF, and this increase was completely inhibited by the PAC1-specific antagonist, PACAP(6–38) (Fig. 2C). The PAC1 ligand, VIP, also increased [^{3}H]thymidine incorporation in NPCs (Fig. 2D) after incubation for 6 h. However, the DNA synthesis-promoting activity of VIP was lower than that of PACAP38 or maxadilan, and was inhibited by PACAP(6–38) (data not shown). These results indicate that activation of PAC1 promotes DNA synthesis in 6 h followed by NPC proliferation in 24 h in cultures supplemented with b-FGF.

To investigate whether PACAP secreted from embryonic cortical NPCs induces DNA synthesis in the presence of b-FGF, we analyzed [^{3}H]thymidine incorporation in NPCs cultured with conditioned medium derived from embryonic cortical NPCs supplemented with b-FGF. After 7 h, [^{3}H]thymidine incorporation increased by 140% relative to NPCs cultured with NPC-free media, and this activity was inhibited by another PAC1-specific antagonist, M65 (Fig. 3). Similar inhibition was observed when PACAP(6–38) was used (data not shown). We also performed a radioimmunoassay to detect PACAP38 in the conditioned medium derived from NPCs. The PACAP concentration was substantially higher in conditioned medium derived from NPCs (284 ± 2.3 pg/mL, Mean ± SEM) than in NPC-free medium (0.072 ± 0.25 pg/mL, Mean ± SEM).

PLC/IP$_3$ Signaling Pathway are Activated by Maxadilan Via PAC1 Splice Variants in Embryonic Cortical NPCs

To determine which splice variant (or variants) was expressed in our embryonic cortical NPCs, we performed RT-PCR using splice variant-specific primer pairs. We detected four PAC1 variants (Fig. 4).

To determine whether PAC1 couples to the cAMP pathway, we measured intracellular cAMP concentration in NPCs after treatment with PACAP38 or maxadilan. Both reagents elicited a 3.5–4-fold increase in intracellular cAMP concentration (Fig. 5A). We used the Fluo-3 ratio method to determine the effects of PAC1 activation on calcium signaling. The intracellular calcium level ([Ca^{2+}]i) increased rapidly in NPCs following treatment with PACAP38 or maxadilan (Fig. 5B). This activity was inhibited by BAPTA-AM, which was an intracellular Ca^{2+}-chelator (Fig. 5B). We used pathway-specific in-

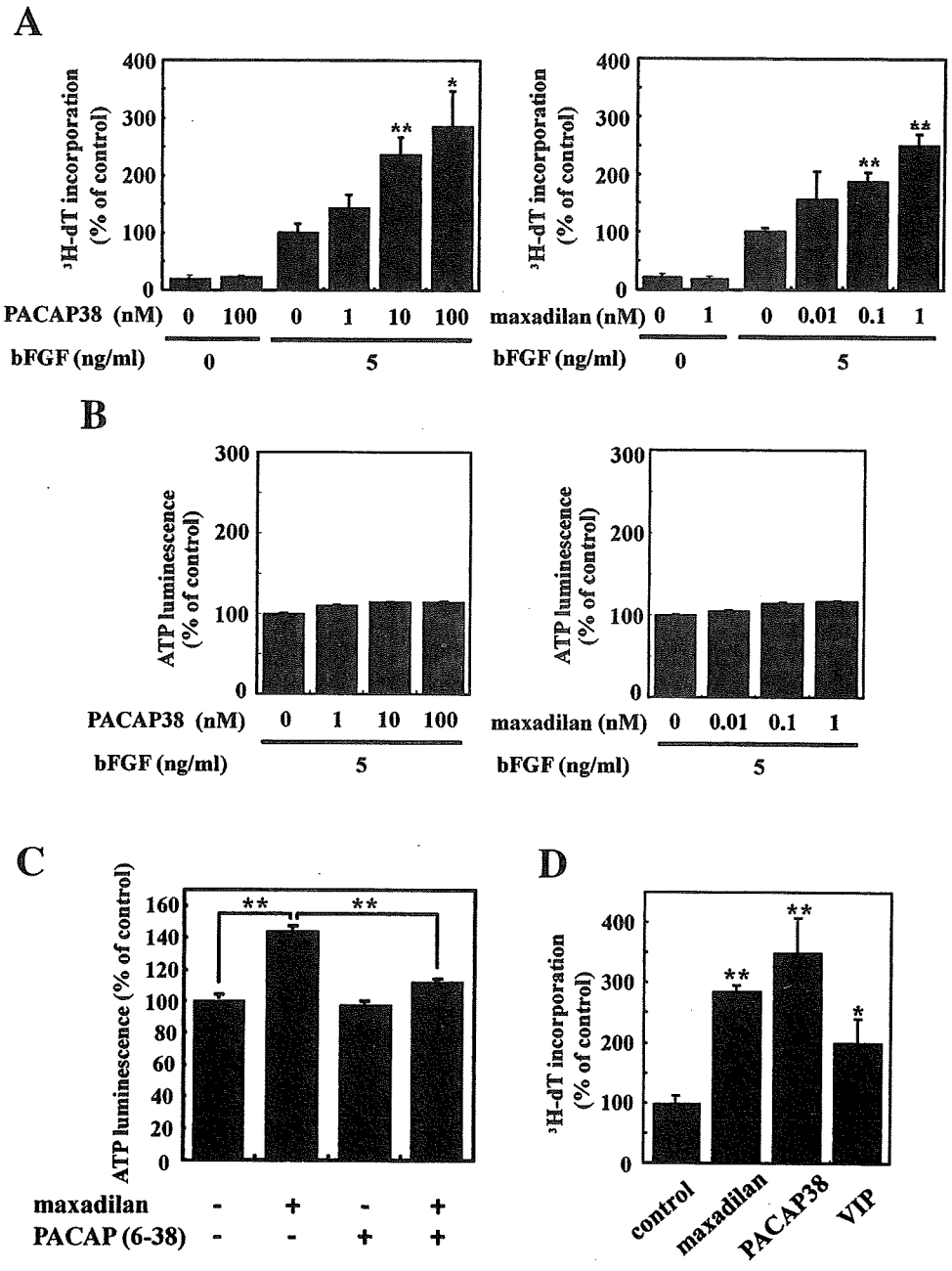

Fig. 2. PAC1 signaling promotes NPC growth in the presence of b-FGF. **A,B:** PACAP38 and maxadilan promote DNA synthesis and cell growth of NPCs in a dose-dependent manner in the presence of b-FGF. NPCs were cultured with PACAP38 (0–100 nM) (left) or maxadilan (0–1 nM) (right) in the presence or absence of b-FGF (5 ng/mL) for 7 h, and then [^{3}H]thymidine was added during the last 6 h of culture (A). NPCs were cultured with PACAP38 (left) or maxadilan (right) in the presence of b-FGF (5 ng/mL) for 6 h, and then the growth rate was examined by an ATP luminescence assay (B). **C:** The PAC1-specific agonist, maxadilan, promotes NPC growth; blockade of PAC1 with the PAC1-specific antagonist, PACAP (6–38), completely cancels this growth-promoting activity. NPCs were incubated with or without maxadilan (1 nM) for 24 h. Maxadilan-induced cell growth was inhibited by co-incubation with PACAP (6–38) (10 nM). **D:** NPCs were cultured with or without PACAP38 (100 nM), maxadilan (10 nM) or VIP (1 μM) in the presence of b-FGF for 7 h, and then [^{3}H]thymidine was added during the last 6 h of culture. Bars represent mean ± SD (n = 4). Significant differences from control (without agonist or antagonist) are indicated by asterisks (*P < 0.05; **P < 0.01, ANOVA).

hibitors for protein kinase A (PKA) or inositol 1,4,5-trisphosphate (IP3)/C kinase (PKC) to determine which signaling pathway mediates PAC1 activity during embryonic cortical NPC proliferation. H89, which inhibits cAMP-dependent PKA, did not inhibit the maxadilan-induced increase in [^{3}H]thymidine incorporation; however, 2-APB, which inhibits the IP3 receptor, had a strong inhibitory effect (Fig. 5C). In addition, chelerythrin, which inhibits PKC, did not inhibit the effect of maxadilan. Taken together, these results indicate that NPC proliferation involved the PLC/IP_3-dependent signaling pathway and a downstream Ca^{2+}-dependent pathway.

PAC1 Activation Induces NPC Proliferation and Morphological Changes in Embryonic Cortical NPCs

Upon maxadilan stimulation for 48 h, we observed a morphological change, which was an elongation of cell processes with stellate and astrocyte-like morphology, in nestin-immunoreactive NPCs (Fig. 6A). To determine the relationship between NPC proliferation and the morphological changes seen by PAC1 signaling, we identified nestin-immunoreactive NPCs in the mitotic phase with a BrdU incorporation assay. Immediately after 10 min of

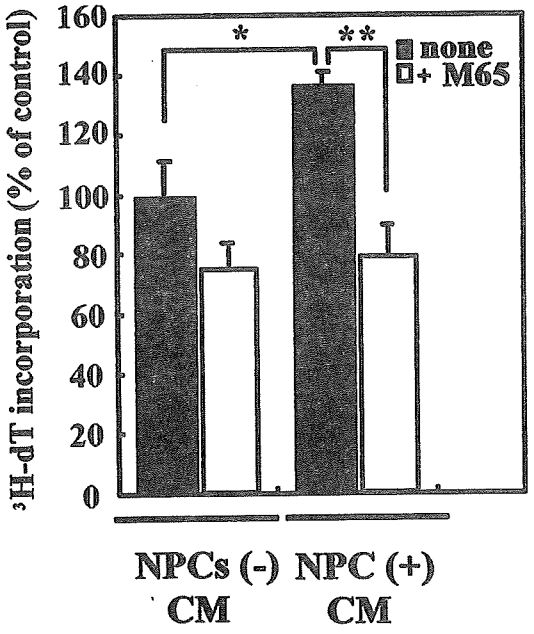

Fig. 3. The activation of PAC1-induced NPC proliferation in autocrine system. NPCs were cultured for 24 h, and the conditioned medium (CM) was collected. The CM or control cell-free media was added to freshly prepared NPC cultures and incubated for 7 h. [^{3}H]thymidine was added during the last 6 h, and incorporation was later measured. DNA synthesis-promoting activity in CM was observed and this activity was inhibited by the PAC1-selective antagonist, M65 (10 nM). Bars represent mean ± SD (n = 4). Significant differences from the control are indicated by asterisks (*P < 0.05, **P < 0.01; ANOVA).

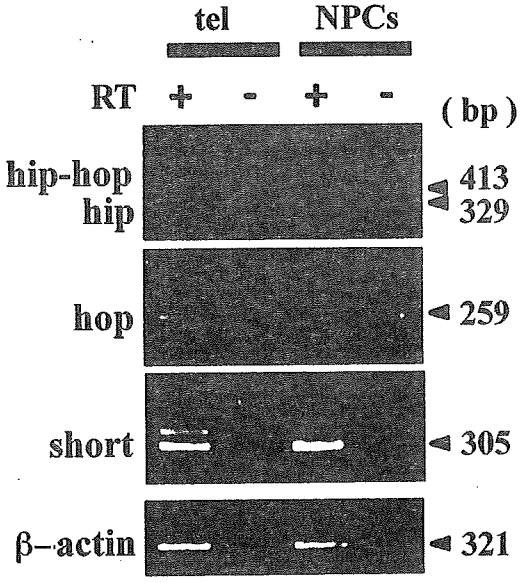

Fig. 4. NPCs express all PAC1 isoforms. RT-PCR was performed as described in Material and Methods. RT-PCR of E14.5 mouse embryonic telencephalon (tel) was used for comparison. The PCR products corresponding to the PAC1 isoforms were loaded onto a 3% agarose gel, as indicated at the left. The size of the PCR products is indicated on the right.

exposure to BrdU, the unincorporated BrdU was washed off the NPCs and maxadilan (10 nM) was added in BrdU-free and b-FGF contained medium. After 24 h, maxadilan increased the number of BrdU/nestin immunoreactive NPCs with long cell processes compared with non-treated NPCs (Fig. 6B). Next, we addressed the differentiation of BrdU immunoreactive NPCs to neurons, astrocytes, or oligodendrocytes. We observed a gradual increase of BrdU immunoreactive NPCs by the 24-h treatment of maxadilan during 7 days, compared with non-treated NPCs. The treatment of maxadilan did not increase the number of β III tubulin immunoreactive neurons, whereas maxadilan induced marked increase of GFAP immunoreactive astrocytes. Moreover, we observed the significant increase in the percentage of BrdU/GFAP double immunoreactive astrocytes, compared with that of non-treated NPCs at day 7 (Figs. 6B–E). These data suggested that PAC1 signaling regulates the proliferation of glial progenitor cells to generate astrocytes in E14.5 NPCs.

DISCUSSION

The PACAP/PAC1 system plays an important role in regulating differentiation of embryonic NPCs at E12–17 (Lee et al., 2001; Lelievre et al., 2002; Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Suh et al., 2001). In our results, the PACAP/PAC1 autocrine system potentiated growth factor-promoted proliferation of E14.5 mouse cortical NPCs. Moreover, we found that the activation of PAC1 initiated morphological changes, which were cell process elongation of typical astrocytes in embryonic cortical NPCs. In the BrdU incorporation assay, most of BrdU positive NPCs differentiated to astrocytes. We suggest dual aspects of PAC1 signaling in the regulation of not only differentiation but also proliferation of NPCs at E14.5 via PLC/IP_3 signaling pathways through PAC1 variants in NPCs committed to an astrocytic lineage.

We found that the PACAP/PAC1 system potentiated growth factor-induced proliferation of E14.5 mouse cortical NPCs in an autocrine manner. Many trophic factor-generated microenvironments control NPC proliferation or differentiation. In particular, growth factors such as b-FGF, EGF, and TGFα promote NPC proliferation in the embryonic and postnatal brain (Gritti et al., 1996; Kilpatrick and Bartlett, 1993; Richards et al., 1992; Vescovi et al., 1999). Although these growth factors are soluble and thus diffuse widely in the CNS, their regulation during development has not been fully understood. In our results, immunoreactivity for PACAP and PAC1 in VZ/SVZ as well as the detection of PACAP38 in conditioned medium derived from NPCs supported the evidence of a PACAP/PAC1 autocrine loop in embryonic NPCs. Several molecules function in an autocrine manner in embryonic NPCs. BMPs and noggin are autocrine factors that regulate NPC proliferation and differentiation (Mabie et al., 1999; Nadarajah et al., 2002; Panchision et al., 2001; Sauvageot and Stiles, 2002). In adult brain, Cystatin C, IGF-I and stem cell-derived stem/progenitor cell-supporting factor have been charac-

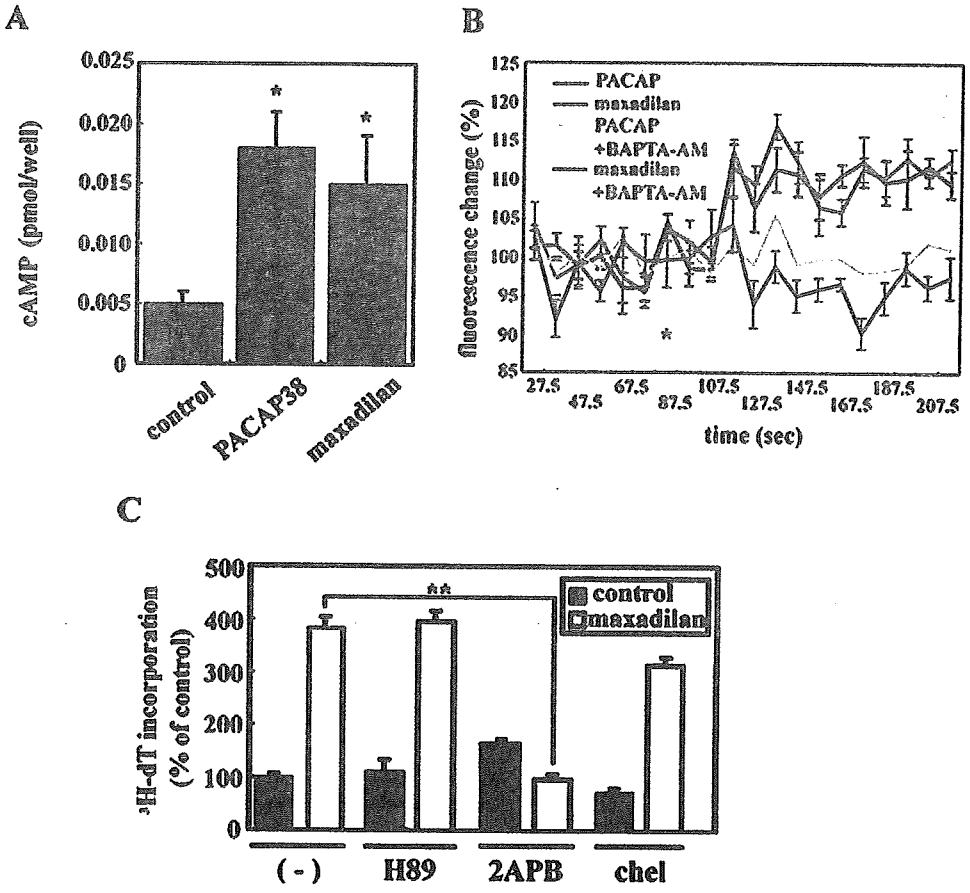

Fig. 5. PACAP promotes DNA synthesis in NPCs via PKC and IP3, but the PAC1-selective agonist, maxadilan, promotes the activity via IP3 only. **A:** The activation of PAC1 increases intracellular cAMP level in NPCs. The intracellular cAMP content in NPCs was measured after treatment with PACAP38 (100 nM) or maxadilan (10 nM) for 15 min by ELISA system. **B:** The activation of PAC1 increases intracellular calcium level ($[Ca^{2+}]i$) in NPCs. The ratio of ($[Ca^{2+}]i$ was analyzed by Fluo-3 AM imaging at the steady state and after the treatment* of PACAP38 (100 nM) or maxadilan (10 nM). **C:** NPCs were incubated with 10 μM H89, 2.5 μM 2-APB or 100 nM chelerythrine in the presence of 5 ng/mL b-FGF for 1 h before the addition of 100 nM PACAP38 or 10 nM maxadilan. [^{3}H]thymidine was added during the last 6 h of culture. Data represent mean values ± SD (n = 4). Significant differences from the control are indicated by asterisks (*P < 0.05, **P < 0.01; ANOVA).

terized as autocrine/paracrine growth-supporting factors for adult NPC proliferation, which is promoted by b-FGF/EGF (Toda et al., 2003). These factors are required to maintain multipotent adult NPC proliferation (Toda et al., 2003). These autocrine systems, including the PACAP/PAC1 system, are considered to play an important role in restricting or amplifying growth factor-mediated signals and in controlling NPC proliferation and differentiation.

At an early developmental stage (E12–14) during which the mouse cortex undergoes neurogenesis, the PACAP/PAC1 system promotes neuronal differentiation of cortical NPCs (Dicicco-Bloom et al., 1998; Lee et al., 2001; Lu and DiCicco-Bloom, 1997; Suh et al., 2001). In contrast, cortical NPCs derived from a late stage (E17) promote astrocyte differentiation via PAC1 (Vallejo and Vallejo, 2002). Furthermore, at the postnatal stage, when oligogenesis occurs, PAC1 signaling regulates both the growth and differentiation of oligodendrocyte progenitor cells (Lee et al., 2001). Thus the PACAP/PAC1 system plays multiple roles in the different cell lineages during development, and this fact suggests that it may function via several signaling pathways. Recent studies have shown that Gs- or Gq-mediated intracellular signal via PAC1 splice variants induced a specific biological activity, differentiation or proliferation, in cortical progenitor cells (Bresson-Bepoldin et al., 1998; Jaworski and Proctor, 2000; Lu et al., 1998; Zhou et al., 2000a,b). Our RT-PCR experiment showed that PAC1 splice variants were expressed in mouse embryonic cortical NPCs at E14.5. Moreover, we detected intracellular cAMP accumulation and $[Ca^{2+}]i$ increase via PAC1 activation in NPCs (Figs. 5A,B). These results suggested that the activation of PAC1 stimulated both Gs-mediated AC/PKA and Gq-mediated PLC/IP3 signaling pathways in embryonic cortical NPCs at E14.5 via PAC1 variants. Although many studies have reported that AC/PKA signal induced NPC differentiation into neuron or glial cells via PAC1 (Zhou et al., 2001), the effects of PLC/IP3 signal in NPC lineage is unknown. We found that IP3 inhibitor curtailed PAC1-mediated NPC proliferation (Fig. 5C), suggesting that Gq-mediated PLC/IP3, not Gs-mediated AC/PKA, signaling pathway modulated NPC proliferation via the PAC1 variant which might give rise to different signaling pathways for NPC differentiation and proliferation.

Cortical NPCs at E14.5 may have heterogeneous subpopulations. Some of these are multipotent and others are committed to neuronal or glial progenitors which generate neurons or astrocytes (Sauvageot and Stiles, 2002). As indicated by our morphological study and BrdU labeling, PAC1 signaling potentiated the pro-

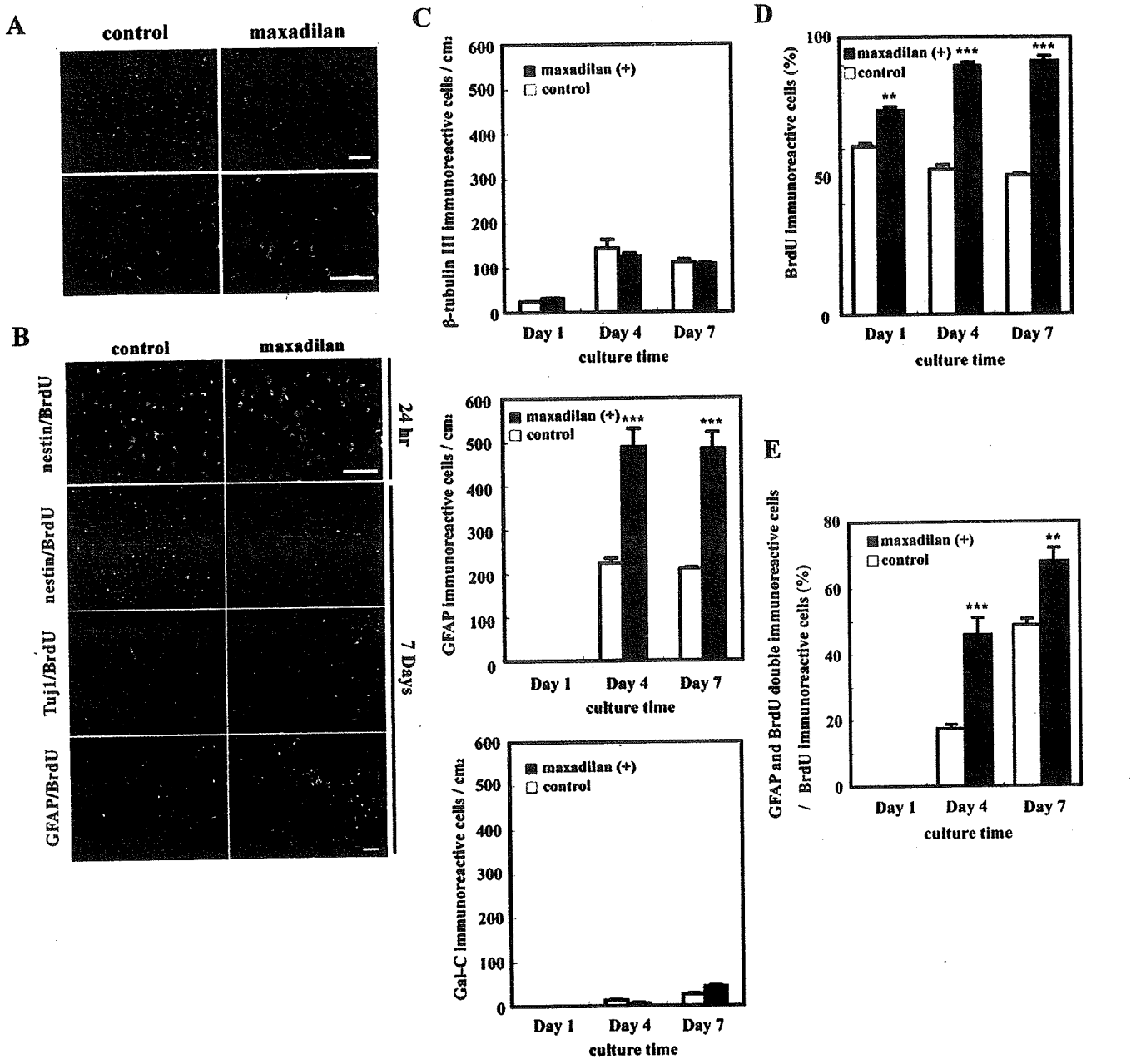

Fig. 6. PAC1 signaling promotes the proliferation of embryonic cortical NPCs committed to astrocytes. **A:** After maxadilan treatment for 24 h, cells were fixed and stained for nestin (green), Hoechst (blue) and BrdU (red). **B:** After maxadilan treatment for 7 days, cells were fixed and stained for nestin (green), β III tubulin (b III tub) (green), GFAP (green), Hoechst (blue) and BrdU (red). **C:** Quantitative analysis of differentiation of embryonic cortical NPCs treated or non-treated with maxadilan for 7 days. **D:** Proliferation of embryonic cortical NPCs treated or non-treated with maxadilan assessed by BrdU incorporation assay. **E:** Time course of astrogenesis from BrdU immunoreactive embryonic cortical NPCs treated or non-treated with maxadilan. Total cellular counts were obtained as described in Materials and Methods. Data represent mean values ± SD ($n = 4$). Significant differences from the control are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ANOVA). Scale bars: (A, B) 50 μm.

liferation of NPCs with longer cell processes manifesting astrocyte-like shapes. Moreover, most BrdU immunoreactive NPCs were differentiated into GFAP immunoreactive astrocytes via PAC1. One of the autocrine factor families, BMPs, are reported to promote astrocytic differentiation and to change the proliferative activities of cortical NPCs in the different developmental stages (Gross et al., 1996; Mabie et al., 1999). Our data may reflect a dual role for the PACAP/PAC1 system; potentiating glial progenitor cell proliferation and subsequent astrogenesis as well as astrocytic differentiation in NPCs similar to BMPs. The PACAP/PAC1 autocrine system is critical for the regulation of NPC and glial lineage, depending on the stage of brain development.

ACKNOWLEDGMENTS

We thank Dr. Richard G. Titus for providing maxadilan and M65. We thank Mr. Ayukawa K. and Mrs. Hara Y. for the early work contribution and excellent technical help.

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Alpha 1-adrenoceptor agonists protect against stress-induced death of neural progenitor cells

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Received 15 February 2007; received in revised form 18 June 2007; accepted 26 June 2007
Available online 12 July 2007

Abstract

Here, we show that α_1-adrenoceptor agonists suppress stress-induced death of mouse embryonic brain-derived neural progenitor cells (NPCs). NPCs highly expressed both α_{1A}- and α_{1B}-adrenoceptor genes, whereas the gene encoding α_{1D}-adrenoceptor was expressed at low levels. Application of the α_1-adrenoceptor agonists phenylephrine and cirazoline significantly promoted cell survival of embryonic NPCs that had been exposed to stress, as measured by a lactate dehydrogenase release assay, but had no remarkable effect on differentiation of the NPCs. Both phenylephrine and cirazoline protected NPCs from death induced by growth factor deprivation, N2 nutrient deprivation, tunicamycin treatment or staurosporine treatment. Phenylephrine and cirazoline treatments both maximally reduced stress-induced cell death by ~60% but did not change the percentage of undifferentiated cells as measured by nestin staining. Moreover, phenylephrine and cirazoline treatments did not affect the cellular activities of caspase-3 and caspase-7 but markedly reduced propidium iodide penetration into the cytoplasm, suggesting that α_1-adrenoceptor agonists inhibit caspase-3/7-independent death of the embryonic NPCs.

Keywords: α_1-adrenoceptor; GPCR; Neural progenitor cell; Cell death; Cell stress; Phenylephrine; Cirazoline

1. Introduction

The noradrenergic system is proposed to play multiple roles in the adult central nervous system (CNS). Apart from its classical transmitter signaling action, noradrenaline has important roles in attention, arousal, and memory reviewed in Murchison et al., (2004); Southwick et al., (1999). Furthermore, it was proposed that noradrenaline influences the survival, maintenance and plasticity of CNS neurons, including the regulation of endogenous neurotrophin systems, glial function, CNS energy utilization and extracellular homeostasis, and has anti-inflammatory and anti-oxidant effects reviewed in Marien et al., (2004). All cell surface adrenoceptors are members of the G protein-coupled receptor family and mediate responses to extracellular noradrenaline. To date, three subfamilies of adrenoceptors (α_1, α_2 and β) have been identified (Bylund et al., 1995). The adrenoceptors are expressed in many tissues, particularly in the cardiovascular, genitourinary and nervous systems. High levels of adrenoceptors are also present in the neocortex during embryogenesis (Lidow and Rakic, 1992), and there are regional concentrations of α_1, α_2, and β adrenoceptors in the fetal forebrain (Lidow and Rakic, 1994). There are three subtypes of α_1-adrenoceptors, the α_{1A}, α_{1B}, α_{1D}-adrenoceptor with varying degrees of efficiency of G protein (Gq/11) coupling ($\alpha_{1A}>\alpha_{1B}>\alpha_{1D}$ adrenoceptor) reviewed in Hieble et al., (1995). This leads to activation of downstream signa

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doi:10.1016/j.ejphar.2007.06.060

transduction pathways, including Ca^{2+}, arachidonic acid, phospholipase C and phospholipase D signals reviewed in Zhong and Minneman (1999). α_1-adrenoceptors are specifically localized to NPCs located in the ventricular zone and subventricular zone in the embryonic rat forebrain, and noradrenaline-containing fibers are also present in both the ventricular zone and subventricular zone (Pabbathi et al., 1997). In addition, noradrenaline has been suggested to regulate development of the murine forebrain. The β-adrenoceptor agonist isoproterenol alters proliferation and differentiation of neural precursors in the cerebral cortex (Slotkin et al., 1988). α_1-adrenoceptors were also implicated in controlling cell proliferation and survival in a rat cortical mixed cell culture and in a heterochronic coculture system of the rat neocortex and rostral pons (Pabbathi et al., 1997; Popovik and Haynes, 2000). Although the functions of α_1-adrenoceptors in the mixed-culture NPCs and in the organ culture were investigated, the function of α_1-adrenoceptors and the direct effects of α_1-adrenoceptor-selective agonists such as phenylephrine and cirazoline in isolated pure embryonic NPCs are not known.

The precise role of the α_1-adrenoceptor-mediated signal in embryonic cortical NPCs remains obscure, because the effects observed in the mixed-cell and organ culture experiments possibly reflect the secondary and tertiary effects mediated by multiple cellular interactions (glial cell-progenitor, neuronal cell-progenitor and neuronal cell-glial cell-progenitor interactions). As such, the aim of this study is to know the primary biological effect of α_1-adrenoceptor activation in the NPCs. We addressed the biological effects of α_1-adrenoceptor-selective agonists on highly purified embryonic NPCs. Our data indicate that these agonists inhibit death of NPCs cultured under various stress conditions but do not affect proliferation, differentiation or caspase-3/7-activity.

2. Materials and methods

2.1. Animals

Pregnant C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Animal care and handling were in accordance with institutional regulations for animal care and public law, and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, Japan.

2.2. Antibodies and reagents

Monoclonal and polyclonal antibodies used in this study were as follows: monoclonal anti-nestin (Becton Dickinson, Lexington, KY), monoclonal anti-tuj1 (Covance, Berkeley, CA), polyclonal anti-glial fibrillary acidic protein (Dako, Carpinteria, CA), monoclonal anti-galactocerebroside (Chemicon International, Temecula, CA). The secondary antibodies conjugated to Alexa Fluor dye were purchased from Molecular Probes (Eugene, OR). α_1-adrenoceptor agonists used in this study were (*R*)-3-[1-hydroxy-2-(methylamino) ethyl] phenol (phenylephrine; Sigma, St. Louis, MO) and 2-[(2-cyclopropylphenoxy) methyl]-4, 5-dihydro-1H-imidazole (cirazoline; Tocris, Ellisville, MO). Each agonist was dissolved in Neurobasal™ medium (Invitrogen, Carlsbad, CA). Staurosporine and tunicamycin (Sigma) were dissolved in dimethyl sulfoxide (DMSO). Each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to at most 0.1% (v/v). Medium containing the same amount of organic solvent was used as a negative control.

2.3. Cortical NPCs culture

Cortical NPCs were cultured as previously described (Fukazawa et al., 2006). Briefly, embryos were removed from pregnant C57BL/6J mice and were staged according to morphological criteria to confirm gestational age. Developing mouse brain and cerebral cortex containing the ventricular and subventricular zones were dissected from embryonic day 14 (E14) embryos. Cells were mechanically dissociated by trituration and plated at 3.0×10^6 cells per 10-cm dish (BD) precoated with 15 μg/ml poly-L-ornithine (Sigma) and 1 μg/ml fibronectin (Nitta Gelatin, Osaka, Japan). Cells were expanded for 4 days in serum-free Neurobasal medium supplemented with 0.5 mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and B27 (biotin, L-carnitine, corticosterone, ethanolamine, D(+)-galactose, glutathione (reduced), linoleic acid, linolenic acid, progesterone, putrescine, retinyl acetate, selenium, T3 (triodo-l-thyronine), DL-α-tocopherol (vitamin E), DL-α-tocopherol acetate, bovine serum albumin, catalase, insulin, superoxide dismutase, transferrin, vitamin A (Brewer et al., 1993); Invitrogen). N2 supplement (100 mg/l apo-transferrin, 5 mg/l insulin, 16 mg/l putrescine, 6.3 μg/l progesterone, 5 μg/l selenite; Sigma) were used for stress experiments instead of the B27 supplement. This medium was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ) except when mentioned otherwise. Cultures were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. For secondary cultures, bFGF-expanded cortical NPCs were washed in warm Hanks' balanced salt solution, detached with mechanically pipetting, and resuspended in Neurobasal medium. Cells were then re-seeded on 24-well plates (Nunc; 1.8×10^5 cells/well); or 48-well plates (Nunc; 1.5×10^5 cells/well) precoated with poly-L-ornithine and fibronectin.

2.4. Real-time quantitative reverse-transcription (RT)-PCR

Real-time quantitative RT-PCR with the SYBR Green-based detection method was performed as previously described (Aoki et al., 2002). Total RNA was isolated from cultured cortical NPCs and E14 mouse cerebral cortex. These RNAs (1 μg) were treated with DNase I and converted to cDNA with Superscript II reverse transcriptase (Invitrogen) and random hexamer primers according to the manufacturer's instructions. The efficiency of reverse transcription and the quality of cDNA was compared with the efficiency of PCR amplification of the hypoxanthine guanine phosphoribosyl transferase (hprt) gene (GenBank accession ID. NM_013556; forward primer, 5′-TCTTTGCTGACCTGCTGGATT-3′; reverse primer, 5′-TATGTCCCCCGTTGACTGATC-

3′). Primers were designed for the α_{1A} (NM_013461), α_{1B} (NM_007416) and α_{1D} (NM_013460) adrenoceptor genes using Primer Express software (Perkin-Elmer, Torrance, CA). The forward and reverse primer sequences were as follows: 5′-TTTCAAGCCACCGGAAACA-3′ and 5′-ACTGGATTCGCAGCACATTCT-3′ (α_{1A}); 5′-AACCCTTCTACGCCCTCTTTTC-3′ and 5′-CCAGATTCTTGGTGGTCCTCTT-3′ (α_{1B}); and 5′-TCGCTCAAGTATCCAGCCATT-3′ and 5′-AACCTAGTAGCGGTCCCACAGA-3′ (α_{1D}). SYBR Green-based real-time RT-PCR was performed in 12.5-μl reactions (ABI PRISM 7700 Sequence Detection System, Perkin-Elmer). PCR products were analyzed with agarose gel electrophoresis. We checked each primer individually to ensure that the primer was selective for the target (data not shown). We also ensured that no band was observed in gel electrophoresis of PCRs that included distilled water or total RNA preparation without reverse transcriptase as template. The quantitative RT-PCR method (User Bulletin #2, Applied Biosystems, Foster City, CA) was modified to establish an expression level index for mRNA (Aoki et al., 2002), and the SYBR Green signal for the hprt amplicon was used as a reference. Amplification efficiency was determined and confirmed in a control PCR experiment using serial cDNA dilutions as templates. The real-time RT-PCR products were analyzed using the Applied Biosystems sequence detection system software 1.7.

2.5. LDH and ATP assay

The number of non-viable cortical NPCs was quantitatively assayed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) released into the culture medium after membrane rupture. LDH activity was measured using the cytotoxicity assay CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI). To quantify the number of viable cells in cultured cortical NPCs, the amount of cellular ATP was measured using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega). These assays were performed in accordance with the manufacturer's protocol and on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated without or with 10 μM phenylephrine or cirazoline with different concentrations of bFGF or under different stress conditions as described in the figure legends. Cultures were then returned to the 37 °C incubator for 24 h, and assessment of LDH release in the media and amount of ATP was conducted with a Wallac 1420 multilabel counter (Perkin-Elmer, Finland).

2.6. Immunocytochemistry

Cells were stained as we have previously described with minor modifications (Sakurai et al., 2006). Briefly, all incubations and washes were performed at room temperature. Cells were fixed with 3.8% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.02% (w/v) Triton X-100 in PBS for 5 min. Fixed cells were blocked with 3.3% goat serum in PBS for 30 min. Cells were incubated for 30 min with anti-nestin (neural progenitor marker 1:500), anti-tuj1 (early neuronal cell marker; 1:500)(Sakura et al., 2006), anti-glial fibrillary acidic protein (astrocyte marker; 1:1000) or anti-galactocerebroside (immature oligodendrocyte marker; 1:200)(Fukazawa et al., 2006). These cells were incubated with diluted secondary antibody (1:200) conjugated to Alexa Fluor for 30 min. All primary and secondary antibodies were diluted in 1% goat serum in PBS before use. The fluorescence microscopy images were obtained with an IX70 microscope (Olympus).

2.7. Quantification of enzymatic activities of caspases

Caspase-3 and caspase-7 protease activities were determined using the Caspase-Glo™ 3/7 Assay kit (Promega). All assays were performed on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated with or without 10 μM phenylephrine in medium lacking the N2 supplement as described in the figure legends. Cultures were then returned to the 37 °C incubator for 24 h, and caspase-3 and -7 activities were assessed with a Wallac 1420 multilabel counter.

2.8. Measurement of cell death using propidium iodide

All assays were performed on secondary cultured cortical NPCs as described above. Four hundred μl (1.5×10^5 cells/well) of cell suspension was added to each well of a 48-well plate (Nunc) precoated with poly-L-ornithine and fibronectin. After 24 h, cells were treated with or without 10 μM phenylephrine in medium lacking the N2 supplement. Cultures were then returned to the 37 °C incubator and maintained for 24 h, then stained with 1 μg/ml propidium iodide. Only dead cells with permeable plasma membranes were stained with propidium iodide. Positive controls were stained with propidium iodide after fixing with 3.8% formaldehyde in PBS for 10 min and permeabilized with 0.02% Triton X-100 in PBS. Dead cells and positive controls stained with propidium iodide were counted by fluorometry (Wallac 1420 multilabel counter).

2.9. Statistical analysis

Results are expressed as the mean±standard error of the mean (S.E.M.). Either the Student's *t*-test or Dunnett's multiple range test was used to evaluate the data using Prism software version 4.03 (GraphPad, San Diego, CA). Values of $P<0.01$ and $P<0.05$ were considered statistically significant depending on the specific experiment.

3. Results

3.1. Embryonic cortical NPCs express α_1-adrenoceptor genes

We analyzed gene expression levels of the three α_1-adrenoceptors, and all were expressed both in the E14 embryonic

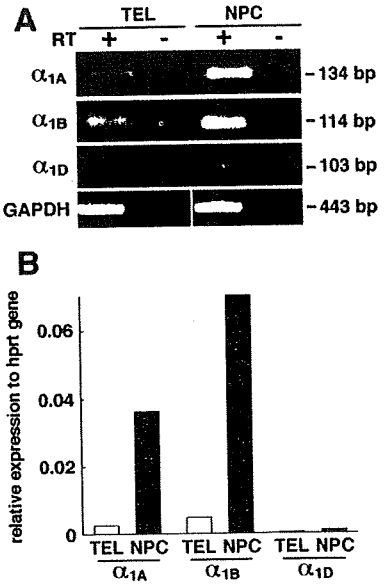

Fig. 1. NPCs derived from E14 telencephalon express α_{1A}-adrenoceptor (α_{1A}), α_{1B}-adrenoceptor (α_{1B}) and α_{1D}-adrenoceptor (α_{1D}). Total RNA isolated from cultured NPCs and E14 mouse telencephalon (TEL) was reverse-transcribed, and the resultant cDNA was used in RT-PCR analysis. (A) As indicated on the left, the PCR products corresponding to the α_{1A}, α_{1B}, and α_{1D} adrenoceptor genes and *GAPDH* were separated on a 3% agarose gel. The size of PCR products are indicated on the right. (B) Gene expression levels of the α_{1A}, α_{1B} and α_{1D} adrenoceptor were analyzed by quantitative RT-PCR, and the expression levels relative to *hprt* are presented. The results are representative of three separate experiments that yielded similar results.

telencephalon and E14 telencephalon-derived cultured NPCs (Fig. 1A). Semi-quantitative analysis of expression levels of the α_1-adrenoceptor genes (normalized to the internal control, *hprt*) showed that the isolated NPCs highly expressed α_{1A}, α_{1B} and α_{1D} adrenoceptor genes as compared with the E14 telencephalon (Fig. 1B). Among the α_1-adrenoceptor genes, the α_{1A} and α_{1B} genes were highly expressed in the cultured NPCs, whereas a low level of expression was detected for the α_{1D} gene in both the E14 telencephalon and cultured NPCs (Fig. 1B).

3.2. Effect of the α_1-adrenoceptor agonist phenylephrine on NPC differentiation

E14 telencephalon-derived NPCs have potencies to differentiate into multiple neural cell types, including neurons, astrocytes and oligodendrocytes, in the absence of bFGF (Fig. 2). The effect of phenylephrine, an α_1-adrenoceptor-selective agonist, on NPC differentiation was examined using the neural cell differentiation marker tuj1 for neuronal cells, glial fibrillary acidic protein for astroglial cells and galactocerebroside for oligodendrocytes. At 72 h after bFGF deprivation, 41.8±1.0% of the NPCs had differentiated into tuj1-positive (tuj1$^+$) cells, 40.6±6.0% of the NPCs had differentiated into glial fibrillary acidic protein-positive (GFAP$^+$) cells and 9.4±1.1% were galactocerebroside-positive (GC$^+$) (Fig. 2A and B). Phenylephrine treatment did not significantly change the percentages of neuronal and glial cells (tuj1$^+$, 38.1±3.2%; GFAP$^+$, 28.7±3.1%; and GC$^+$, 6.2±1.0%) in cultures lacking bFGF (Fig. 2A and B). These results indicate that phenylephrine did not affect NPC differentiation induced by bFGF deprivation. We also examined the effect of phenylephrine on neural differentiation of NPCs in cultures containing bFGF and again found no effect on neuronal or glial differentiation (data not shown).

3.3. α_1-adrenoceptor agonists protect NPCs from cell death

It has been reported that activation of α_1-adrenoceptors stimulates DNA synthesis of embryonic NPCs in mixed culture conditions (Pabbathi et al., 1997). Primary NPC cultures prepared from the telencephalon contain a considerable number of neuronal cells (>10%) that also express α_1-adrenoceptors (Papay et al., 2006). To determine whether the previously reported activation of DNA synthesis was indicative of NPC proliferation or proliferation by secondary effect via other cell types contaminating the culture, we re-seeded cultured NPCs from a primary culture to prepare highly purified secondary

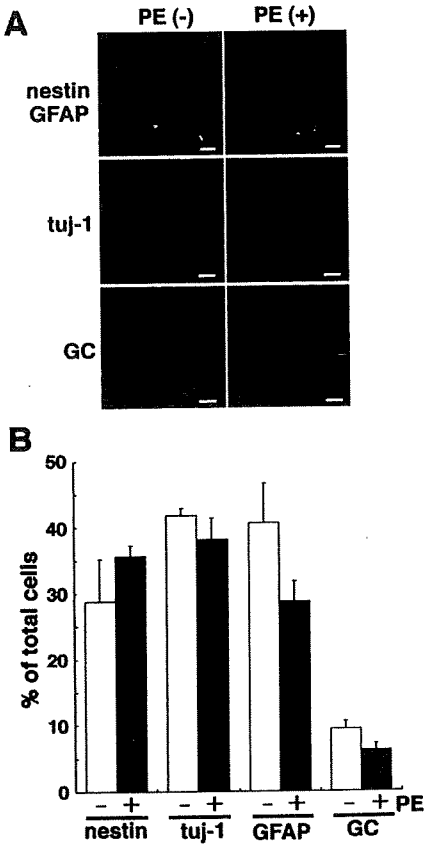

Fig. 2. Effect of the α_1-adrenoceptor agonist phenylephrine on NPC differentiation. (A) Immunofluorescence staining was carried out after 72 h on NPC cultures with or without phenylephrine. Fluorescence microscopic images of cells labeled with anti-nestin (green), anti-GFAP (red), anti-tuj1 (green) and anti-galactocerebroside (green) are shown; nuclei are stained with Hoechst (blue). Scale bar=20 μm. Similar results were obtained in two independent experiments. (B) Secondary cultured NPCs from the E14 mouse telencephalon were maintained *in vitro* for 72 h without or with 10 μM phenylephrine (PE). After 72 h, cells were fixed and immunostained for tuj1, glial fibrillary acidic protein (GFAP), nestin and galactocerebroside (GC). The number of nestin$^+$, tuj1$^+$, GFAP$^+$ and GC$^+$ cells were counted, and the percentages are presented. Nestin was used as a marker for undifferentiated NPCs. No significant differences were observed.

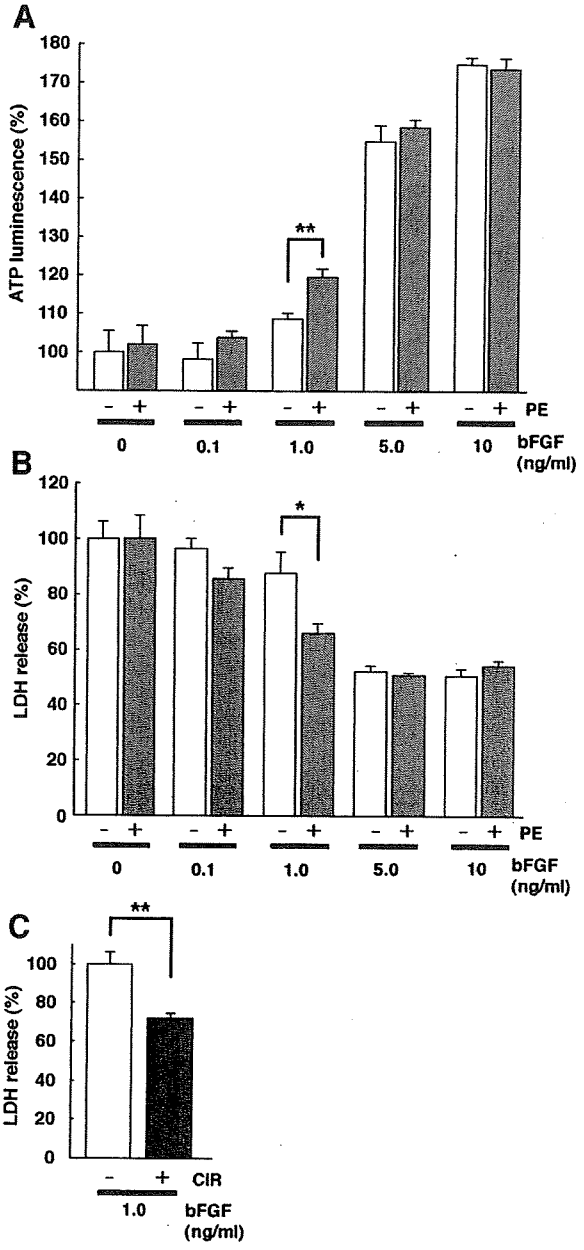

Fig. 3. Effects of α_1-adrenoceptor agonists on the number of cells and cell death of NPCs at various doses of bFGF. Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM phenylephrine at different concentrations of bFGF for 24 h. (A) The number of NPCs was examined with an ATP luminescence assay. (B) The viability of NPCs was examined with the LDH release assay. (C) Secondary cultured NPCs from the E14 mouse telencephalon were treated without or with 10 μM cirazoline in 1 ng/ml bFGF for 24 h. Cell survival was assessed by the ATP luminescence assay. Bars represent mean±S.E.M. (n=4). Significant differences are indicated by single or double asterisks (*P<0.05, **P<0.01, Student's t-test).

NPC cultures that contained over 99±0.4% nestin$^+$ undifferentiated NPCs and no more than 0.5% tuj1$^+$ neuronal cells. Using the secondary NPC culture, we examined whether phenylephrine increased the number of NPCs at various doses of bFGF (0~10 ng/ml) using an intracellular ATP luminescence assay (Crouch et al., 1993; Petty et al., 1995). We found that phenylephrine significantly increased the number of NPCs only at a moderate dose (1.0 ng/ml) of bFGF (P<0.01; Fig. 3A) and had no significant effect at high doses (5–10 ng/ml), a low dose (0.1 ng/ml) of bFGF, or no bFGF. To determine whether the effect of phenylephrine on NPCs at 1 ng/ml bFGF was due to promotion of cell growth or cell survival, we examine the effect of phenylephrine on cell death using the LDH release assay (Decker and Lohmann-Matthes, 1988), which measures destruction of the plasma membrane. Phenylephrine also significantly decreased LDH release at 1 ng/ml bFGF (P<0.05; Fig. 3B), indicating that the difference between ATP luminescence (cell numbers) of phenylephrine-treated and

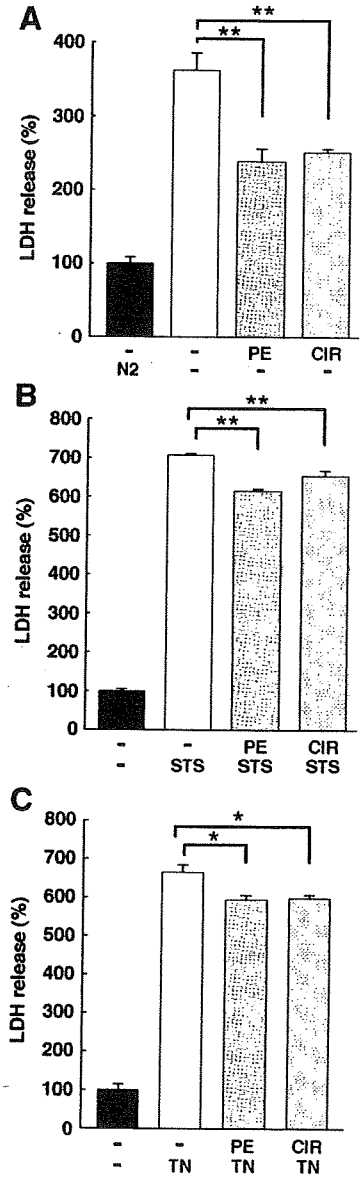

Fig. 4. Phenylephrine and cirazoline promote survival of NPCs under various stress conditions. Secondary cultured NPCs from the E14 mouse telencephalon were incubated in medium lacking N2 (A), 50 nM staurosporine (STS) (B) or 30 ng/ml tunicamycin (TN) (C) in the presence or absence of 10 μM phenylephrine (PE) or 10 μM cirazoline (CIR) for 24 h. Quantification of cell death was performed with the LDH release assay. Bars represent mean±S.E.M. (n=3~5). Significant differences are indicated by single or double asterisks (*P<0.05, **P<0.01, Dunnett's test).

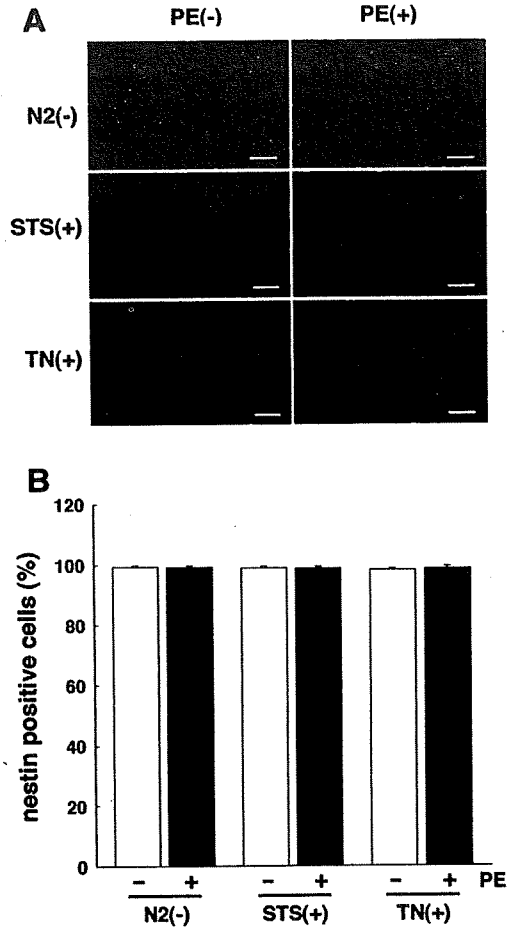

Fig. 5. Effect of phenylephrine on the proportion of nestin-positive cells under various stress conditions. Secondary cultured NPCs were exposed for 24 h to stress conditions without or with 10 μM phenylephrine: N2 deprivation medium, or exposure to 50 nM staurosporine (STS) or 30 ng/ml tunicamycin (TN). After 24 h, the NPCs were fixed and stained with anti-nestin and Hoechst. (A) Fluorescence microscopic images of cells labeled with anti-nestin (green) and Hoechst (blue). Scale bar=50 μm. (B) The percentages of nestin-positive cells in the cultures were quantified. No significant differences were seen among the groups.

untreated NPCs correlated with the difference in the amount of cell death. These results also indicated that phenylephrine protected NPCs from death during bFGF deprivation-induced stress. Moreover, we confirmed that α_1-adrenoceptor agonists specifically protected NPCs from death using another α_1-adrenoceptor agonist, cirazoline. Cirazoline treatment of NPCs under the same culture conditions resulted in a significant decrease in LDH release ($P<0.01$; Fig. 3C), indicating that α_1-adrenoceptor agonists promote survival of NPCs cultured in 1 ng/ml bFGF. A [^{3}H]thymidine incorporation assay showed that phenylephrine and cirazoline did not induce DNA synthesis of NPCs (data not shown).

3.4. α_1-adrenoceptor agonists prevent NPC death upon exposure to various stresses

To determine the extent to which α_1-adrenoceptor agonists could prevent NPC death, we employed other stress conditions: N2 deprivation, or exposure to 50 nM staurosporine or 30 ng/ml tunicamycin. The results of the LDH release assay for these stress conditions showed that N2 deprivation, staurosporine treatment, and tunicamycin treatment induced LDH release from NPCs (361±24%, 706±5% and 664±21%, respectively, relative to the controls; Fig. 4). However, application of the agonists to the NPC cultures under these stress conditions significantly reduced LDH release (N2 deprivation+phenylephrine, 238±18% $P<0.01$; N2

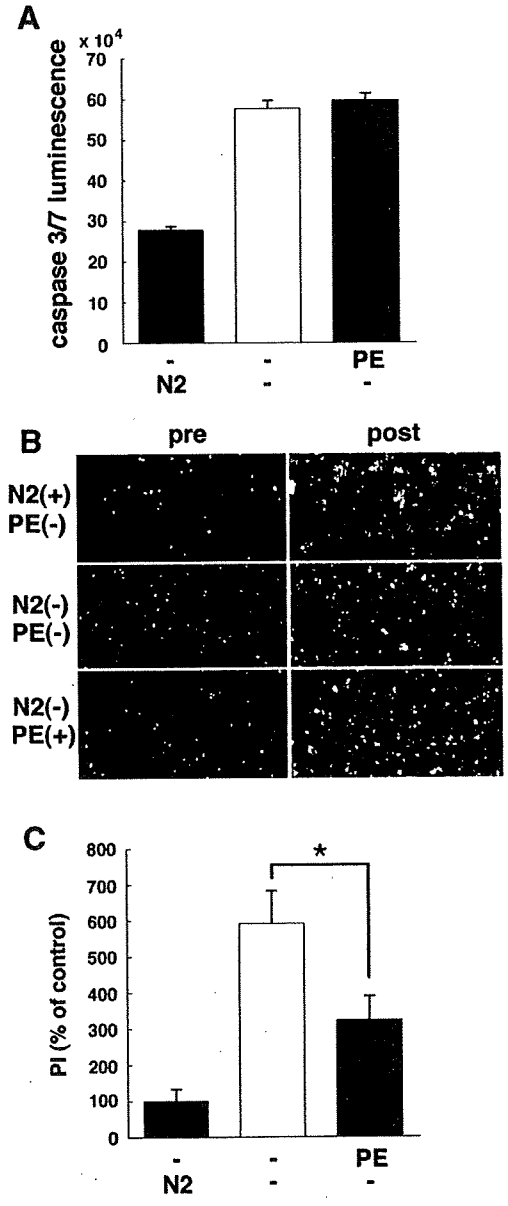

Fig. 6. Phenylephrine does not alter cellular caspase activity but changes propidium iodide penetration into NPCs. Secondary cultured NPCs were incubated without or with 10 μM phenylephrine (PE) under N2 deprivation conditions for 24 h. (A) Caspase-3 and caspase-7 activities in cell lysates were measured by luminometry. (B) Fluorescence microscopy images of dead cells stained with propidium iodide are presented (left). For positive controls, cells were fixed and stained with PI after permeabilization with 0.02% Triton X-100 (right). Scale bar=100 μm. (C) The intensity of propidium iodide staining was measured by fluorometry. Bars represent mean±S.E.M. ($n=4$). Significant differences are indicated by an asterisk (*$P<0.05$, Dunnett's test).

deprivation+cirazoline, 251±5% $P<0.01$; staurosporine+phenylephrine, 614±9% $P<0.01$; staurosporine+cirazoline, 654±16% $P<0.01$; and tunicamycin+phenylephrine, 594±13% $P<0.05$; tunicamycin+cirazoline, 597±10% $P<0.05$) (Fig. 4). Under the three stress conditions, the differences in the efficacies of phenylephrine and cirazoline on LDH release did not differ greatly. However, both agonists were most effective against N2 deprivation stress, where phenylephrine and cirazoline reduced LDH release 34% and 31%, respectively, as compared with the unstressed control (Fig. 4A). These data suggest that α_1-adrenoceptor agonists protected NPC death under several different stress conditions but exhibited different efficacies depending on the particular stress.

3.5. α_1-adrenoceptor agonists do not modulate the proportion of nestin-positive NPCs under stress conditions

Phenylephrine did not affect differentiation of NPCs in cultures with or without bFGF (Fig. 1), and the high proportion (over 98%) of nestin-positive cells remained for ~24 h even in 1 ng/ml bFGF (data not shown). The ability of the agonists to prevent cell death in 1 ng/ml bFGF was thus a direct effect on the nestin-positive NPCs and not an artifact due to contaminating cells. We also examined the effects of stress induced by N2 deprivation, 50 nM staurosporine or 30 ng/ml tunicamycin on NPC differentiation to exclude the possibility that the increased cell numbers observed following α_1-adrenoceptor agonist exposure, as measured by ATP production, were derived from the differentiated cells induced by the stresses. The proportion of nestin-positive NPCs was not changed by N2 deprivation, staurosporine-or tunicamycin-induced stress, and treatment of NPCs with phenylephrine or cirazoline under these stress conditions did not decrease the proportions of nestin-positive cells (Fig. 5).

3.6. Phenylephrine inhibits caspase-3/7-independent cell death

Of the three stress conditions tested, α_1-adrenoceptor agonists most effectively suppressed stress caused by N2 deprivation, as measured by LDH release (Figs. 3 and 4). Whereas cell death induced by bFGF deprivation, staurosporine treatment or tunicamycin treatment probably was caused by activation of various death signaling pathways, the stress caused by N2 deprivation mainly induced caspase-3/7-dependent cell death in NPC cultures (Fig. 6). To ascertain whether α_1-adrenoceptor agonists protect against caspase-3/7-dependent cell death, we examined the effects of α_1-adrenoceptor agonists on cellular caspase-3/7 activities. Stress caused by N2 deprivation induced elevated cellular caspase-3 and caspase-7 activities as compared with non-stress conditions (>2 fold; Fig. 6A). However, the elevated activities of caspases were not changed by treatment of NPCs with phenylephrine (Fig. 6A). Despite a lack of modulation of the cellular caspase activity, phenylephrine suppressed propidium iodide penetration into NPC cytoplasm (45.4%; $P<0.05$, as compared with untreated cells deprived of N2) (Fig. 6B and C). These data indicate that α_1-adrenoceptor agonists selectively protect against caspase-3/7-independent death of NPCs exposed to stress.

4. Discussion

In this study, we prepared highly purified embryonic NPCs (>99% nestin$^+$ cells) from the E14 mouse cortex and found that the cortical embryonic NPCs highly express α_{1A}- and α_{1B}-adrenoceptor genes but express the α_{1D}-adrenoceptor gene at low levels. Our pharmacological experiments also revealed that α_1-adrenoceptor agonists are protective against NPC death induced by various stresses without any modification of the cell differentiation state of the NPCs. Moreover we demonstrated that α_1-adrenoceptor agonists reduced NPC death caused by the N2 deprivation stress without modulation of intracellular caspase-3/7 activities.

The α_1-adrenoceptor is expressed in the ventricular zone and subventricular zone of the embryonic rat forebrain (Pabbathi et al., 1997). The ventricular zone of the embryonic cerebral cortex contains both undifferentiated NPCs and differentiated nascent neuronal cells. Our quantitative RT-PCR analysis showed that purified cortical NPCs express α_{1A}- and α_{1B}-adrenoceptor genes at high levels compared with the E14 embryonic telencephalon, suggesting that the undifferentiated NPCs highly expressed the α_{1A}- and α_{1B}-adrenoceptor genes. We could not confirm the expression of α_1-adrenoceptor proteins in NPCs because of low specificity of commercially available antibodies against α_1-adrenoceptor in immunocytochemical experiments with NPCs (data not shown). However, we demonstrated that α_1-adrenoceptor agonists have protective effects against cell death in NPCs, indicating the presence of the α_1-adrenoceptors in NPCs.

The chemical structure of phenylephrine ((*R*)-3-[1-hydroxy-2-(methylamino) ethyl] phenol) differs from that of cirazoline (2-[(2-cyclopropylphenoxy) methyl]-4, 5-dihydro-1H-imidazole). However, both α_1-adrenoceptor agonists had the same effect on NPC death induced by a moderate concentration (1 ng/ml) of bFGF, nutritional deprivation (no N2 supplementation), staurosporine treatment or endoplasmic reticulum stress (tunicamycin treatment), indicating that the effects were specifically mediated by α_1-adrenoceptors. It is well known that cell death can be induced via multiple apoptosis signaling pathways that are specifically activated by different stresses. Although the α_1-adrenoceptor agonists were able to protect NPCs from death induced by the stresses we tested, the molecular mechanism that underlies this broad protection is unknown. However, treatment of NPCs with α_1-adrenoceptor agonists failed to decrease the activities of caspase-3 and caspase-7, which are activated in the apoptosis pathway. Instead, α_1-adrenoceptor agonists reduced propidium iodide incorporation induced by stress caused by N2 deprivation. These data suggest that α_1-adrenoceptor agonists protect against necrotic NPC death but not apoptotic NPC death.

Phenylephrine or cirazoline protects against cell death induced by bFGF deprivation as well as by STS and nutritional deprivation, or treatment with staurosporine or tunicamycin. Niidome et al. (Niidome et al., 2006) showed that NPC death resulting from growth factor deprivation is caused by both caspase-dependent and -independent pathways in concert with oxidative stress, suggesting that α_1-adrenoreceptor agonists may also protect against NPC death induced by oxidative stresses. On the other hand, application of phenylephrine is effective only against cell death induced by 1.0 ng/ml bFGF. Complete

deprivation of growth factors activates multiple cell death pathways, including apoptosis, necrosis and the oxidative cell death pathway, in cultured NPCs (Niidome et al., 2006). Therefore, the application of phenylephrine is unlikely to be effective against NPC death induced by the overlapping activation of multiple death pathways after complete loss of growth factor support.

In a recent study, Hiramoto et al. (2006) reported that the stimulation of α_1-adrenoreceptors by phenylephrine or by L-epinephrine induces the proliferation of NPCs derived from cultured neurospheres. Our data indicate that α_1-adrenoreceptor agonists do not induce proliferation of purified NPCs. It is known that the neurospheres have the 3D organization in which nestin-positive (progenitor) cells surround a large core of differentiated GFAP-positive (glial) and β-tubulin III-positive (neuronal) cells (Campos, 2004). As such, the NPC cultures prepared from neurospheres could also contain glial and neuronal cells. Thus, the difference between these data may have resulted from differences in the NPC preparation methods and from the purity of the nestin-positive NPCs.

Transplantation of neural stem and progenitor cells into patients with intractable neurological diseases is considered an effective strategy for neural regeneration therapy reviewed in Lindvall et al., (2004). Studies on cell transplantation/implantation for CNS disorders have indicated that neural stem cells and progenitor cells have the ability to replace lost neurons and to repair the damaged nervous system (Chu et al., 2004). However, a large proportion of grafted cells is lost due to early necrotic death. Thus, the low rate of graft survival reduces the effectiveness of such therapies (Emgard et al., 2003). Here we demonstrated that α_1-adrenoceptor agonists protect against NPC death (probably necrotic death) induced by various stresses. We also demonstrated that α_1-adrenoceptor agonists with this protective effect did not modify the cell differentiation state of the NPCs. Thus, α_1-adrenoceptor agonists may be useful for the preparation and maintenance of neural stem and progenitor cells for transplantation therapy, as they are likely to increase cell viability without induction of unexpected cell differentiation. Moreover, phenylephrine is a popular and safe drug that is used as a non-prescription decongestant (Chua and Benrimoj, 1988) Therefore, phenylephrine may be easily applied as an additional reagent in the cultivation medium of neural stem and progenitor cells for transplantation therapy.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and a grant from Japan Science and Technology Agency.

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Facilitation of Extinction Learning for Contextual Fear Memory by PEPA: A Potentiator of AMPA Receptors

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Behavioral/Systems/Cognitive

Facilitation of Extinction Learning for Contextual Fear Memory by PEPA: A Potentiator of AMPA Receptors

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Contextual fear memory is attenuated by the re-exposure of mice to the context without aversive stimulus. This phenomenon is called extinction. Here, we report that a potentiator of AMPA receptors, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA), potently facilitates extinction learning in mice. C57BL/6J mice were exposed to novel context and stimulated by electrical footshock. After 24 h (extinction training) and 72 h (extinction test), the mice were repeatedly exposed to the context without footshock and the duration of their freezing response was measured. The duration of freezing response in the extinction test was consistently shorter than the value in extinction training. Intraperitoneal injection of PEPA 15 min before extinction training remarkably reduced the duration of freezing responses during the extinction training and test, compared with the vehicle-injected control mice. This action of PEPA on extinction was dose-dependent and inhibited by NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide), an AMPA receptor antagonist. PEPA had no effect on acquisition and consolidation of fear memory itself. Electrophysiological studies suggested that PEPA activates the neural network much more potently in the medial prefrontal cortex (mPFC) than in the basolateral amygdala and hippocampal CA1 field. Quantitative PCR studies suggested the pronounced expression of PEPA-preferring AMPA receptor subunits (GluR3 and GluR4) and a splice variant (flop) in the mPFC. An intra-mPFC injection of PEPA facilitated the extinction much more potently than an intra-amygdala injection of PEPA did. These results suggest that PEPA facilitates extinction learning through AMPA receptor activation mainly in the mPFC.

Key words: AMPA receptor; potentiator; PEPA; extinction; fear; medial prefrontal cortex

Introduction

Contextual conditioned fear is acquired in response to an aversive stimulus such as electrical shock [unconditioned stimulus (US)] that is loaded during exposure of mice to a conditioned stimulus (CS), such as novel context. Re-exposure to the CS triggers a conditioned response (CR) such as defensive freezing behavior (for review, see Fanselow 1998; LeDoux 2000; Davis and Whalen, 2001; Maren 2001). However, if the CS is presented without the US, the CR gradually declines. This phenomenon is called "extinction" of conditioned fear memory (for review, see Myers and Davis, 2002; Maren and Quirk 2004). It is thought that extinction is a result of new learning that forms "inhibitory association" between the CS and the US, which suppresses the emergence of the CR (Bouton et al., 1993; Berman and Dudai, 2001; Bouton, 2002; for review, see Myers and Davis, 2002). Connections between the medial prefrontal cortex (mPFC) and the amygdala are thought to be neural bases for extinction (Morgan et al., 1993; Morgan and LeDoux, 1995; Garcia et al., 1999, Quirk et al., 2000; Rosenkranz and Grace, 2001; Quirk et al., 2003).

Studies using positron emission tomography show dysfunction of the medial and orbital PFC during provocation of post-traumatic stress disorder (PTSD) symptoms (Bremner, 1999). Given that the mPFC plays a crucial role in extinction, a drug that could activate the mPFC has the potential to facilitate exposure-based cognitive behavioral therapy (CBT) for psychiatric disorders, such as PTSD, because CBT depends on the process of fear extinction (for review, see Davis et al., 2006). D-Cycloserine, an agonist and potentiator of the NMDA subtype of glutamate receptors, facilitates extinction learning for fear memory (Walker et al., 2002). This compound is thought to facilitate inhibitory association between the CS and US within the amygdala (Walker et al., 2002; for review, see Davis et al., 2006). The AMPA receptor is another major subtype of glutamate receptors and mediates fast excitatory synaptic transmission in the majority of the central glutamatergic synapses (Hestrin et al., 1990; Ozawa et al., 1991). Each AMPA receptor subunit exists in flip and flop variants generated by alternative splicing, and their expression is regulated both regionally and developmentally (Sommer et al., 1990). The activation of AMPA receptors releases NMDA receptors from voltage-dependent magnesium block (Mayer et al., 1984); the role of AMPA receptors in extinction learning, however, is obscure.

In the present study, we investigated the effects of 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide

Received Sept. 5, 2006; revised Nov. 17, 2006; accepted Nov. 21, 2006.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and a grant from the Japan Science and Technology Agency.

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DOI:10.1523/JNEUROSCI.3842-06.2007

 0270-6474/07/270158-09$15.00/0

(PEPA), a flop splice variant and GluR3/4-prefrerring potentiator of AMPA receptors (Sekiguchi et al., 1997, 1998), on extinction learning. Potentiators for AMPA receptors are small chemical compounds that enhance AMPA receptor channel activity by modulating receptor kinetics such as desensitization and deactivation [in the case of PEPA (Sekiguchi et al., 2002)]. Some of these compounds reportedly enhance synaptic response by acting on AMPA receptors [in the case of PEPA (Nakagawa et al., 1999)] (for review, see O'Neill et al., 2004). Here, we found that PEPA potently facilitates extinction learning through activation of AMPA receptors mainly in the mPFC.

Materials and Methods

Animals. Male C57BL/6J mice were purchased from Clea (Tokyo, Japan) and used in experiments after habituation to the environment of our Institute's animal center for 1 week. The mice were housed five per cage under controlled conditions of temperature (25 ± 1°C) and lighting (12 h light/dark cycle) and provided with food and water *ad libitum*. The mice were 8–12 weeks old at the beginning of experiments. The experiments were performed in strict accordance with the regulations of the National Institute of Neuroscience (Japan) for animal experiments.

Behavioral procedures. Contextual fear conditioning was performed using the method reported previously (Takeda et al., 2003) with slight modifications. Briefly, a mouse was introduced to the operation box (20 × 20 cm bottom, 35 cm high, inside a soundproof box) with a stainless grid floor. After 10 s, electrical footshock was delivered from the grid for 360 s (1.3 mA; 1 s duration; 10 s interval; scrambled). The similar stimulus condition, in particular, a condition where multiple electrical shocks are given, is also used in the study to examine the involvement of the mPFC in behavioral control against stress (Amat et al., 2005). Ten seconds after the end of electrical shock, the mouse was removed from the box and returned to his home cage. The behavior of the mouse was recorded using a digital video camera on the ceiling of the soundproof box. The illumination on the floor of the box was 230 lux. Complete immobilization of the mouse, except for respiration, was regarded as the freezing response (Blanchard and Blanchard, 1972). The freezing response was scored as the total time the mouse spent in freezing during the 360 s test session. The total time the mouse spent in freezing during each 60 s of the 360 s test session was plotted for the time-course analysis. The timing of exposure of mice to the operation box varied between experiments and is described in Results. PEPA (Sigma, St. Louis, MO, or kindly supplied from Nihonsuian, Japan) was dissolved into the vehicle consisting of 0.45% NaCl and 33% (2-hydroxypropyl)-β-cyclodextrin, and injected intraperitoneally (0.01 ml/g body weight) into the mice unless otherwise noted. 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX) disodium salt (Sigma) was dissolved in saline (0.9% NaCl). The timing of the injection varied between experiments and is described in Results.

Open-field tests were performed in an arena (a 50 × 50 cm white field surrounded by a 40-cm-high white wall, illuminated with 80 lux) that was placed in the same soundproof box described above. Mice were placed at the periphery of the arena, and for 5 min the behavior of mice was recorded into a computer using a digital video camera. Locomotion and the time the mice spent at a perimeter (i.e., within 10 cm of the wall) were calculated from this record by Image OF (O'Hara, Tokyo, Japan), a modified software based on the public domain of NIH Image.

The hole-board test was performed using the method reported previously (Takeda et al., 1998) with slight modifications. The hole-board apparatus (50 × 50 cm gray floor with four holes 3 cm in diameter, equally spaced, and a 40 cm-high wall, illuminated with 170 lux) was placed in the soundproof box. Mice were placed in the center of the hole board, allowed to freely explore the apparatus for 5 min, and head-dipping behavior was counted by monitoring the mouse behavior using a digital video camera on the ceiling of the soundproof box.

Electrophysiology. Each male mouse (8–12 weeks old) was anesthetized with halothane and the brain was removed quickly. When recordings were performed from the mPFC or basolateral amygdala (BLA), coronal brain slices (300 μm thick) were prepared using a Vibratome 3000 (Vibratome, St. Louis, MO) in artificial CSF [ACSF; containing the following (in mM): 125 NaCl, 4.4 KCl, 1.5 $MgSO_4$, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$, pH 7.4, 290–300 mOsm/l]. When recordings were performed from the CA1 field, the hippocampus was isolated from the brain and transverse slices (400 μm thick) were prepared as described previously (Nakagawa et al., 1999; Takamatsu et al., 2005) using a Vibratome 3000 in a sucrose-based cutting solution [containing the following (in mM): 234 sucrose, 25 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 26 $NaHCO_3$, 11 glucose, 0.5 $CaCl_2$]. The slices were maintained at room temperature in ACSF continuously bubbled with 95% O_2/5% CO_2. A slice was transferred to the recording chamber and was continuously perfused (3 ml/min) with ACSF maintained at 28–32°C. Somatic whole-cell patch-clamp recordings were made with an EPC-9 (HEKA, Lambrecht/Pfalz, Germany) amplifier for the neurons identified using infrared differential contrast videomicroscopy (Hamamatsu Photonics, Hamamatsu, Japan) with an upright microscope (Axioscop; Zeiss, Oberkochen, Germany). In the mPFC slices, the layer V pyramidal-shaped neurons with thick primary apical dendrites toward the midline direction were selected for the recording. The location of the recorded neurons was verified using a brain atlas (Paxinos and Franklin, 2001) and confirmed to be in the regions of the cingulate and prelimbic cortices. The pyramidal-shaped neurons in the BLA and CA1 pyramidal layer were selected for recording. In all three regions, the neurons that were selected for recording showed the spike-frequency adaptation when suprathreshold depolarization pulses were applied. The patch electrodes (6–10 MΩ) were filled with solution containing the following (in mM): 132 K gluconate, 3 KCl, 10 HEPES, 0.5 EGTA, 1 $MgCl_2$, 12 sodium phosphocreatine, 3 ATP magnesium salt, 0.5 GTP, pH 7.4, with KOH, 285–290 mOsm/l. Because the reversal potential of $GABA_A$ receptor-mediated synaptic currents was estimated between −75 and −85 mV when this pipette solution was used, we used a holding potential of −80 mV in the whole-cell clamp mode to minimize the contribution of $GABA_A$ currents in the synaptic response. The signal was digitized at 1 point/50 μs and stored using Pulse/Pulsefit (HEKA). The resting membrane potential of the cells used in the analysis was between −57 and −75 mV and the series resistance was 3–20 MΩ. Input resistance was continuously monitored during recordings (every 30 s), and cells with a large drift in the value (more than ±15% from the initial value) were removed from the analysis. Electrical stimulation was applied to elicit synaptic currents through a metal bipolar electrode positioned on layer II (mPFC), external capsule (BLA), or stratum radiatum on the border of the CA2 and CA3 fields (CA1). The stimulation was performed with a constant current mode (20–100 μs duration). All drugs used in electrophysiological experiments were applied to slices by perfusion. PEPA was dissolved in dimethylsulfoxide (DMSO) at 2, 10, 50, and 150 mM and diluted with ACSF to prepare 2, 10, 50, and 150 μM solutions on the day of use (the final DMSO concentration was 0.1%). Cyclothiazide (Sigma) solution was prepared similarly. These drugs were applied for 10 min during continuous monitoring of synaptic currents (interstimulus interval, 30 s). The 5–10 responses just before the application, during minutes 5–10 from the start of the application and after the application, were respectively averaged, and these traces were compared. To assess the effects of PEPA, we measured the area under the baseline (AUB; ms/pA) using AxoGraph version 3 (Molecular Devices, Union City, CA), and the effect was expressed as an "AUB ratio," which was obtained by dividing the AUB value during the application of PEPA by the value before the application of PEPA. Other chemicals, including CNQX, were purchased from Sigma.

Quantitative PCR. To isolate RNA from the mPFC, we first prepared coronal brain slices (300 μm thickness) including the mPFC (near the bregma +2.3 mm level). The region of the mPFC (mainly consisting of the prelimbic, medial orbital, and cingulate cortices) was manually isolated from slices under a stereoscopic microscope during which the slices were incubated in ACSF continuously bubbled with 95% O_2/5% CO_2. In the case of the amygdala, we also prepared similar brain slices including the BLA (near the bregma −1.7 mm level). The delta region lying between the external capsule (mainly consisting of the BLA and LA) was cutoff from the slices and was saved for isolation of RNA. For the hippocampus, it was isolated from the brain, and transverse slices (400 μm