

Activation of Adenosine A₁ Receptor–Induced Neural Stem Cell Proliferation via MEK/ERK and Akt Signaling Pathways

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Adenosine, a modulator of neuronal function in the mammalian central nervous system, exerts a neuroprotective effect via the adenosine A₁ receptor; however, its effect on neural stem cells (NSCs) remains unclear. Because adenosine is released in response to pathological conditions and NSCs play a key role in neuroregeneration, we tested the hypothesis that adenosine is capable of stimulating NSC proliferation. We demonstrated that NSCs dominantly express adenosine A₁ and A_{2B} receptors. Adenosine and the adenosine A₁ receptor agonist cyclopentyladenosine (CPA) increased proliferation of NSCs, and this CPA-induced cell proliferation was attenuated by the A₁ antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPA). CPA also induced phosphorylation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase/ERK kinase (MEK), and Akt, and their phosphorylation was inhibited by DPCPA. In addition, CPA-induced cell proliferation was inhibited by MEK and Akt inhibitors. These results suggest that activation of adenosine A₁ receptor–stimulated proliferation of NSCs occurs via MEK/ERK and Akt signaling pathways. © 2008 Wiley-Liss, Inc.

Key words: neural stem cells; adenosine A1 receptor; N6-cyclopentyladenosine

Multipotent neural stem cells (NSCs) derived from the central nervous system (CNS) exhibit the properties of self-renewal and the ability to differentiate into neurons, astrocytes, and oligodendrocytes (Gage et al., 1995; Doetsch et al., 1999; Alvarez-Buylla et al., 2001; Bédard and Parent, 2004). NSCs are present in a developing CNS and play an essential role in neural development (Götz and Huttner, 2005). In addition, NSCs play a key role not only in the maintenance of the adult CNS but also in the ability to recover from injury and disease (Lois and Alvarez-Buylla, 1993; Morshead et al., 1998; Johansson et al., 1999). NSCs are activated in response to a variety of pathological states in Parkinson's disease and multiple sclerosis, as well as in brain injuries such as ischemia, trauma, and epilepsy. They can respond to

injury by proliferation and differentiation, although the response is not sufficient to overcome the injury (Dietrich and Kempermann, 2006; Mazurová et al., 2006; Miller, 2006). Therefore, it is essential to study the neurotrophic factors and other endogenous molecules that are enhanced in response to these pathological conditions and that activate NSCs and to understand their signaling pathways.

Adenosine is a ubiquitous purine nucleoside. It is released from metabolically active cells and is generated extracellularly by degradation of released ATP. Extracellular adenosine exerts its action by interacting with four receptor subtypes (A₁, A_{2A}, A_{2B}, and A₃ receptors), which are seven-membrane-spanning proteins, and with G proteins to access several intracellular signaling pathways (Ralevic and Burnstock, 1998; Fredholm et al., 2001). Adenosine A₁ and A₃ receptors are coupled to G_i protein, which results in inhibition of the production of cAMP. In contrast, adenosine A_{2A} and A_{2B} receptors are coupled to G_s protein, which increases the production of cyclic AMP (Ralevic and Burnstock, 1998; Fredholm et al., 2001). Therefore, the biological action of adenosine is dependent on the relative expression and signaling function of the individual adenosine receptor subtypes as well as the extracellular concentration of adenosine. All adenosine receptor subtypes are expressed in the brain; however, their expression is localized in specific sites and cell types (Kreisberg et al., 1997; Fredholm et al., 2001; Lopes et al., 2003). Adenosine is released in large

Additional Supporting Information may be found in the online version of this article.

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quantities during acute CNS insults such as ischemia and trauma (Clark et al., 1997; Weigand et al., 1999; Laghi et al., 2000; Robertson et al., 2001). Furthermore, adenosine kinase, which removes adenosine via phosphorylation of AMP, is down-regulated at the injured brain sites, thereby increasing extracellular adenosine levels (Lynch et al., 1998).

Adenosine exerts neuroprotective influences, such as reduction of glutamate release, interference with vesicle release machinery, activation of presynaptic channels, and inhibition of the NMDA receptor (Brundege and Dunwiddie, 1997; Schubert et al., 1997; Haas and Selbach, 2000). Adenosine exerts its protective actions largely via the adenosine A₁ receptor through a combination of pre- and postsynaptic effects on neuronal functions. Adenosine A₁ receptor activation by selective adenosine A₁ agonists results in an increase in neuroprotective effects; in contrast, blockage of the adenosine A₁ receptor by selective A₁ antagonists results in increased CNS damage (Sweeney, 1997; Johansson et al., 2001). In addition, pharmacological inhibition of the adenosine A₂ receptor by A₂ antagonists produces neuroprotection because A₂ receptor signals oppose A₁ receptor signals (Dixon et al., 1997). Therefore, the adenosine A₁ receptor is suggested to play a pivotal role during acute insults to the CNS.

Given that adenosine is released in response to pathological conditions and that NSCs play a key role in the regeneration of neural cells, we tested the hypothesis that adenosine A₁ receptor signals are capable of NSC proliferation.

MATERIALS AND METHODS

Reagents and Antibodies

Adenosine, N⁶-cyclopentyladenosine (CPA), 2-hexynyl-5-ethylcarboxamidoadenosine (HE-NECA), 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (Cl-IB-MECA), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Sigma (St. Louis, MO). The following adenosine receptor agonists were also purchased from Sigma: adenosine amine congener, N⁶-cyclohexyladenosine, (2S)-N⁶-(2-endo-norbornyl)adenosine (PD 126280), (R)-N⁶-(1-methyl-2-phenylethyl)adenosine, N⁶-2-phenylethyladenosine, N⁶-phenyladenosine, 2-chloroadenosine, 5'-N-(ethylcarboxamido)adenosine (NECA), 5'-(N-cyclopropyl)-carboxamidoadenosine (PD 125944), N-[(2-methylphenyl)methyl]-adenosine (metrifudil), 5'-N-methylcarboxamidoadenosine (MECA), 2-phenylaminoadenosine, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680), N⁶-(4-aminobenzyl)-N-methylcarboxamidoadenosine (AB-MECA), N⁶-benzyl-5'-N-ethylcarboxamidoadenosine (N⁶-benzyl-NECA), and N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA). API-2 (Akt inhibitor) and U0126 (MEK inhibitor) were obtained from Tocris Bioscience (Ellisville, MO) and Calbiochem (San Diego, CA), respectively. Anti-ERK1/2 (#4695), MEK1/2 (#9122), Akt (#9272), anti-phospho-MEK1/2 (Ser217/221; #9154), phospho-ERK1/2 (Thr202/204; #9106), and phospho-Akt

(Ser473; #4051) were purchased from Cell Signaling Technology (Beverly, MA). AlexaFluor 680-labeled antimouse IgG and IRDye 800-labeled antirabbit IgG were purchased from Invitrogen (La Jolla, CA) and Rockland Immunochemicals (Philadelphia, PA), respectively.

Neural Stem Cells

All treatments of animals were conducted according to the guidelines for care and use of laboratory animals of the Research Center Kobe of Bayer Yakuhin (Kobe). NSCs were isolated from the subventricular zones (SVZs) of 9-week-old C57BL/6 mice (Oriental Yeast, Tokyo, Japan) as previously described (Reynolds et al., 1992). In brief, adult mouse brains were dissociated into single cells by trypsin digestion, and the cells were suspended in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12; Invitrogen) containing 100 µg/mL human transferrin (Sigma), 20 nM progesterone (Sigma), 100 µM putrescine (Sigma), 30 nM sodium selenite (Sigma), and 5 µg/mL insulin (Sigma). The suspended cells were plated in tissue culture dishes at a density of 5×10^3 cells/mL. The cells were maintained in an undifferentiated proliferative state by culturing them as free-floating neurospheres in DMEM/F12 containing 100 µg/mL human transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, 5 µg/mL insulin, 20 ng/mL human basic fibroblast growth factor (bFGF; Sigma), and 20 ng/mL epidermal growth factor (EGF; Sigma).

Reverse-Transcriptase Polymerase Chain Reaction Analysis

NSCs were grown to subconfluent monolayers on six-well plates that were coated with ornithine-laminin (ORN/LN). Then total RNA was isolated from NSCs using an RNeasy Mini Kit (Qiagen). For RT-PCR and quantitative RT-PCR analysis, 1 µg of RNA was reverse-transcribed with SuperScript III (Invitrogen), and PCR was performed with 100 ng of cDNA and TaqMan PreAmp Master Mix Kit (Applied Biosystems, Foster City, CA). The specific primer sets for the target genes were: for the adenosine A₁ receptor, Mm01308023_m1; for the A_{2A} receptor, Mm00802075_m1; for the A_{2B} receptor, Mm00839272_m1; for the A₃ receptor, Mm00802076_m1 (all from Applied Biosystems). For the positive control, mouse brain RNA prepared from 9-week-old C57BL/6 mouse brains using an RNeasy Mini Kit, expressed sequence tag clones (A₁ receptor, clone 30536680; A_{2A} receptor, clone 30242398; A_{2B} receptor, clone 40103279; Open Bio Systems, Huntsville, AL), and a RIKEN full-length cDNA clone A₃ (F630018K21; DNAFORM, Yokohama, Japan) were used. PCR was carried out by incubating the reaction mixture for 40 cycles of 15 sec at 95°C and 60 sec at 60°C. For quantitative RT-PCR analysis, expression of adenosine receptor mRNA was estimated as the copy number compared with that of each cDNA.

Cell Proliferation Assay

Cell proliferation was analyzed by either the BrdU incorporation assay or the cell viability assay. For analysis of

bromodeoxyuridine (BrdU) incorporation, NSCs were plated as a monolayer on ORN/LN-coated 96-well plates at a density of 2×10^3 cells/well with DMEM/F12 medium containing 100 $\mu\text{g}/\text{mL}$ human transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 0.05 $\mu\text{g}/\text{mL}$ insulin, 20 ng/mL bFGF, and 2 ng/mL EGF. After 24 hr of culture, either adenosine receptor agonists, antagonist, or dimethyl sulfoxide (DMSO; 0.1% final, for control) was added, and the cells were cultured for 3 days. Then incorporation of BrdU was analyzed according to the manufacturer's protocol (Cell Proliferation ELISA; Roche Diagnostics, Nutley, NJ). In brief, the BrdU solution was added to the cells for the last 2 hr. Then the cells were fixed and incubated with peroxidase-conjugated anti-BrdU antibodies and substrate solution, and the resulting absorbance was measured. For the cell viability assay, NSCs were plated on ORN/LN-coated 96-well plates at a density of 1.5×10^4 cells/well with DMEM/F12 medium containing 100 $\mu\text{g}/\text{mL}$ human transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenite, and 5 $\mu\text{g}/\text{mL}$ insulin. After 24 hr of culture, adenosine receptor agonists were added, and the cells were cultured for 6 days. The number of cells was measured using the CellTiter 96 Aqueous One Solution Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Western Blot Analysis

NSCs were plated on an ORN/LN-coated 6-well plate at 5×10^5 cells/well with DMEM/F12 medium containing 100 $\mu\text{g}/\text{mL}$ human transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 5 $\mu\text{g}/\text{mL}$ insulin, and 20 ng/mL bFGF. After 2 days of incubation, the cells were cultured in growth factor-free medium for 24 hr. The cells were incubated with 0, 100, or 1000 nM DPCPX for 30 min prior to stimulation with the adenosine receptor agonist (100 nM CPA, 100 nM HE-NECA, or 100 nM CI-IB-MECA) for 10 or 60 min. Control samples received dimethyl sulfoxide to 0.1%. The cells were lysed in sodium dodecyl sulfate (SDS)-sample loading buffer (Red Loading Buffer Pack; Cell Signaling Technology). Lysate was loaded onto 10% SDS gel electrophoresis gels (Invitrogen) and blotted to a poly(vinyl difluoride) membrane (Immobilin-FL, Millipore, Billerica, MA), as recommended by the manufacturer. Membranes were blocked with Blocking-One P (Nacalai Tesque, Kyoto, Japan) for 1 hr and incubated with primary antibodies against MEK1/2, phospho-MEK1/2 (Ser217/221), ERK1/2, phospho-ERK1/2 (Thr202/204), Akt, and phospho-Akt (Ser473). After washing, the membranes were incubated with secondary antibodies against AlexaFluor 680-labeled antimouse IgG or IRDye 800-labeled antirabbit IgG. Fluorescent bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Statistical Analysis

Quantitative data are expressed as means \pm SDs of 3–5 experiments. Significance was determined by analysis of variance, followed by the Dunnett test for multiple comparisons. A value of $P < 0.05$ was considered significant.

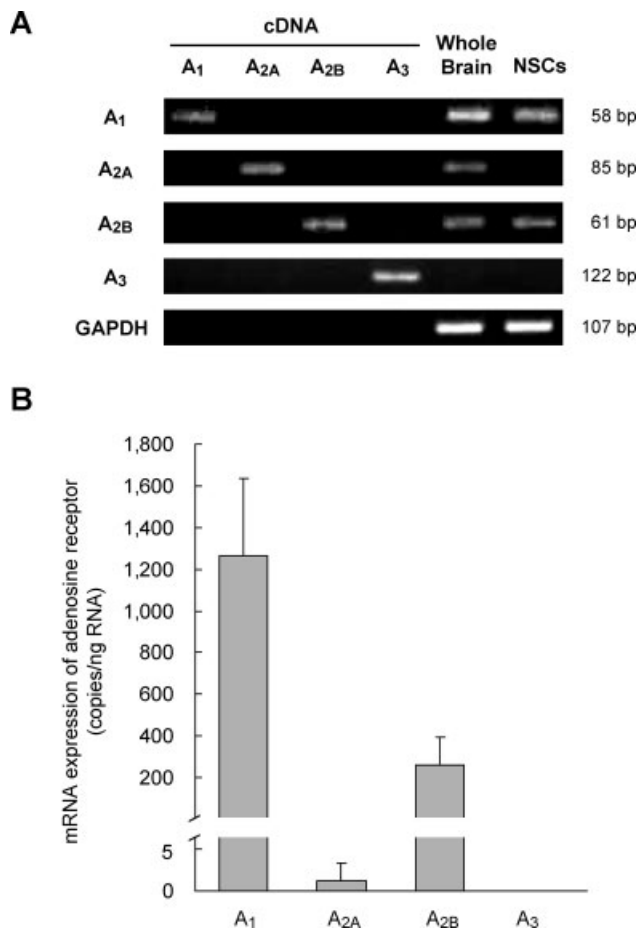


Fig. 1. NSCs dominantly express adenosine A_1 and A_{2B} receptors. **A:** Total RNA from NSCs and mouse whole brain was analyzed by RT-PCR. For the control, cDNA of A_1 , A_{2A} , A_{2B} , and A_3 receptors was used. **B:** Total RNA from NSCs was analyzed by quantitative real-time RT-PCR. mRNA expression of the adenosine receptor is presented as the copy number per 1 ng of total RNA. For the standard control, cDNA of the A_1 , A_{2A} , A_{2B} , and A_3 receptors was used. A_1 , A_{2A} , A_{2B} , and A_3 indicate adenosine A_1 , adenosine A_{2A} , adenosine A_{2B} , and adenosine A_{3A} receptors, respectively.

RESULTS

NSCs Dominantly Express Adenosine A_1 and A_{2B} Receptors

All adenosine receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3) are expressed in the rodent brain. A_1 receptor expression has been detected in many brain regions, and the expression of other subtypes is localized (Kreisberg et al., 1997; Fredholm et al., 2001; Lopes et al., 2003). To understand the biological relevance of adenosine receptors in NSCs, we first investigated the expression of adenosine receptors in the mouse brain and in NSCs by RT-PCR analysis. Expression of A_1 , A_{2A} , and A_{2B} receptors was detected in total RNA from mouse whole brain (Fig. 1A). The A_3 receptor was not detected in mouse brain, although its primer sequences completely

match those of A₃ receptor cDNA, consistent with a low copy number of the A₃ receptor in total RNA from whole brain. Expression of mRNA for the A₁ and A_{2B} receptors was detected in NSCs isolated from the SVZ of mouse adult brains. To compare their expression, quantitative real-time RT-PCR analysis was performed using A₁, A_{2A}, A_{2B}, or A₃ receptor cDNA as each standard control; their mRNA expression was estimated as copy number per 1 ng of total RNA (Fig. 1B). Expression of adenosine A₁ receptor mRNA was prominent and was 4.8-fold that of the adenosine A_{2B} receptor. Expression of adenosine A_{2A} receptor mRNA was detected but was 3 orders less than that of the A₁ and A_{2B} receptors.

Adenosine Stimulates Proliferation of Neural Stem Cells

Based on previous descriptions of the relevant increase in extracellular adenosine in the CNS under various pathological conditions (Clark et al., 1997; Weigand et al., 1999; Laghi et al., 2000; Robertson et al., 2001) and the expression of the adenosine A₁ and A_{2B} receptors by NSCs (Fig. 1), we hypothesized that activation of adenosine receptors activates NSCs. Consistent with this hypothesis, treatment of NSCs with adenosine significantly increased cell proliferation in time- and concentration-dependent manners (Fig. 2).

Activation of Adenosine A₁ Receptor Stimulates Proliferation of Neural Stem Cells

To identify the receptor responsible for mediating adenosine-induced NSC proliferation, we next examined which selective adenosine agonists affected proliferation of NSCs. The selective A₁ agonist CPA (Merighi et al., 2001; Gao et al., 2007) increased cell proliferation in a concentration-dependent manner (Fig. 3A). CPA treatment increased the number of NSCs (Fig. 3B). However, it is difficult to estimate if the A_{2B} receptor directly affected proliferation of NSCs because selective A_{2B} agonists were not available. In contrast, the data on the expression of adenosine receptors in NSCs (Fig. 1) was paralleled because HE-NECA and CI-IB-MECA, which are agonists of the A_{2A} receptor (Merighi et al., 2001) and the A₃ receptor (Merighi et al., 2001), did not affect cell proliferation. In addition, CPA-induced proliferation of NSCs was completely inhibited by 100 nM DPCPX, an A₁ receptor antagonist (Merighi et al., 2001; Fig. 4).

There have been reports of many adenosine receptor agonists, whose selectivity is varied (Merighi et al., 2001; Gao et al., 2007). To further understand the selective effect of the adenosine receptor on NSC proliferation, we tested various adenosine receptor agonists. As expected, all A₁ agonists tested significantly stimulated NSC proliferation (Fig. 5). In contrast, neither A_{2A} agonists nor A₃ agonists affected NSC proliferation.

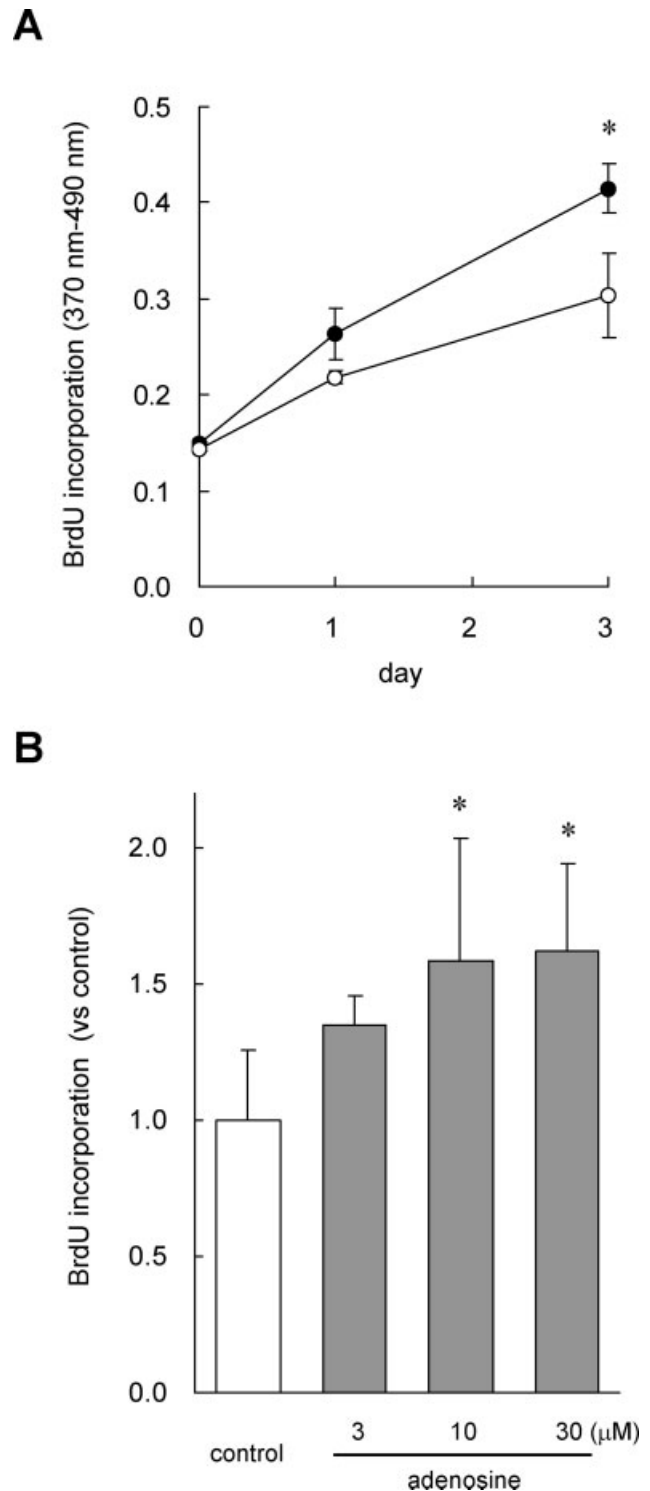


Fig. 2. Adenosine increases proliferation of neural stem cells. **A:** NSCs were treated with μM adenosine (closed circle) or DMSO (open circle) for 3 days, and incorporation of BrdU was measured. **B:** NSCs were treated with adenosine (3, 10, or 30 μM) or DMSO (control) for 3 days, and incorporation of BrdU was measured on day 3. Results are presented as the magnitude of increase relative to the control (* $P < 0.05$ vs. control).

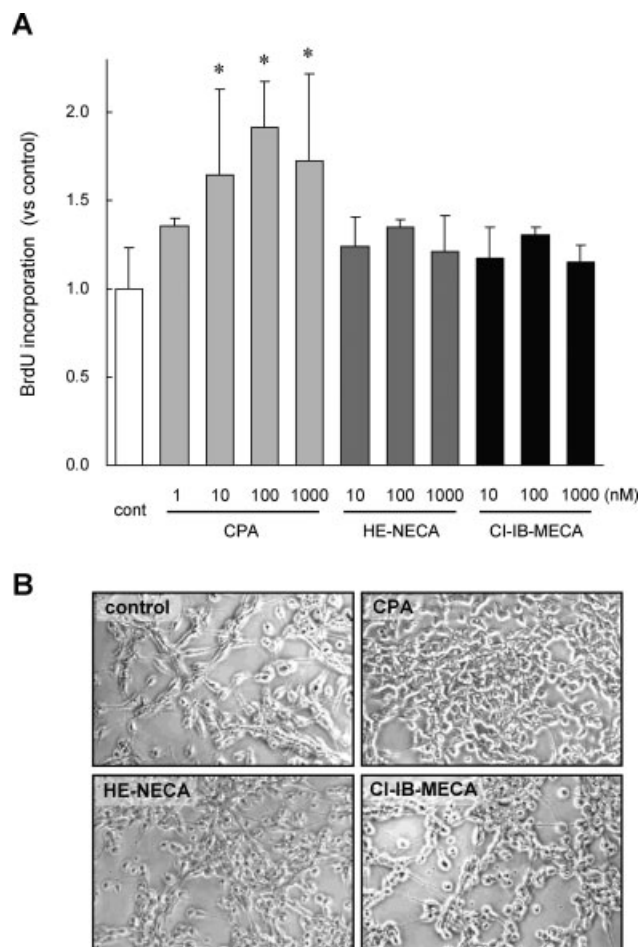


Fig. 3. CPA induces proliferation of neural stem cells. **A**: NSCs were treated with CPA (1, 10, 100, or 1000 nM), HE-NECA (10, 100, or 1000 nM), CI-IB-MECA (10, 100, or 1000 nM), or DMSO (control) for 3 days, and incorporation of BrdU was measured. Results are presented as the magnitude of the increase relative to the control. **B**: NSCs were treated with CPA (100 nM), HE-NECA (100 nM), CI-IB-MECA (100 nM), or DMSO for 3 days ($*P < 0.05$ vs. control).

Activation of Adenosine A₁ Receptor Induces MEK, ERK, and Akt Phosphorylation

Adenosine receptor signaling pathways in neural cells such as neurons and astrocytes have been studied. Activation of the A₁ receptor inhibits cAMP production mediated by coupling to the G_i protein; then it inhibits cAMP-dependent protein kinase A (PKA) activity, which results in inhibition of glutamate release and neuronal activity (Ralevic and Burnstock, 1998; Fredholm et al., 2001). In other cell types such as cardiomyocytes and vascular cells, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and Akt are activated by adenosine A₁ receptor activation (Shen et al., 2005; Peart and Headrick, 2007). Based on these observations, we next investigated whether activation of the adenosine A₁ receptor is linked to MAPK and Akt signaling path-

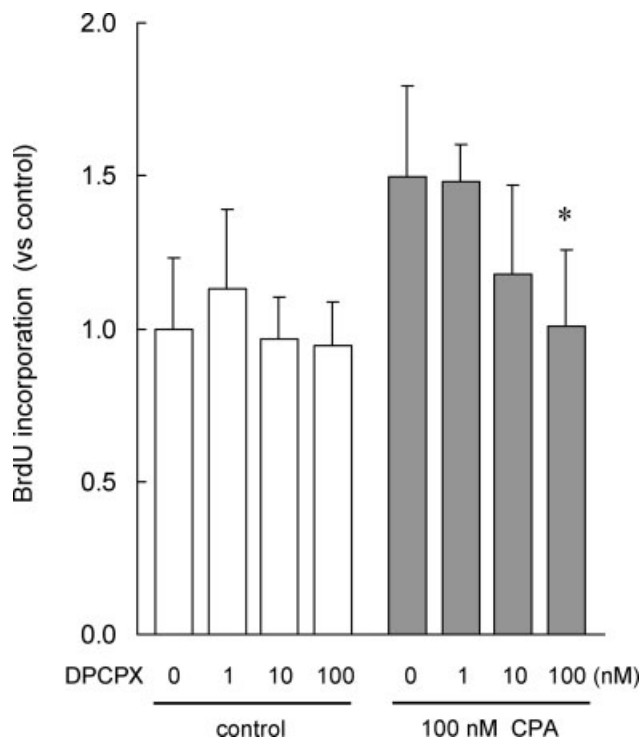


Fig. 4. DPCPX inhibits CPA-induced proliferation of neural stem cells. NSCs were treated with CPA (100 nM) or DMSO (control) in the presence of DPCPX (0, 1, 10, or 100 nM) for 3 days, and then BrdU incorporation was measured. Results are presented as the magnitude of the increase relative to the control without DPCPX ($*P < 0.05$ vs. 100 nM CPA without DPCPX).

ways in NSCs. The treatment of NSCs with 100 nM CPA rapidly increased the phosphorylation levels of extracellular signal-regulated kinase-1/2 (ERK1/2) and MAPK/ERK kinase-1/2 (MEK1/2), which are major kinases of MAPK signaling pathways; this phosphorylation occurred rapidly, in between 5 and 10 min, and decreased gradually 60 min after treatment (Fig. 6A). Similar observations were previously reported in smooth muscle cells (Shen et al., 2005). In addition, treatment of NSCs with 100 nM CPA increased phosphorylation of Akt. In contrast, neither the A_{2B} agonist HE-NECA nor the A₃ agonist CI-IB-MECA induced phosphorylation of MEK1/2, ERK1/2, and Akt. Furthermore, CPA-induced phosphorylation of MEK1/2, ERK1/2, and Akt was inhibited by the pretreatment of NSCs with the selective A₁ antagonist DPCPX (Fig. 6B). Together, these results indicated that activation of the adenosine A₁ receptor stimulated both the MEK/ERK and Akt signaling pathways.

Inhibition of MEK and Akt Activities Attenuated A₁ Agonist-Induced Cell Proliferation

To determine if MEK/ERK and Akt signaling pathways are directly involved in NSC proliferation, we examined the effects of MEK and Akt activities on the

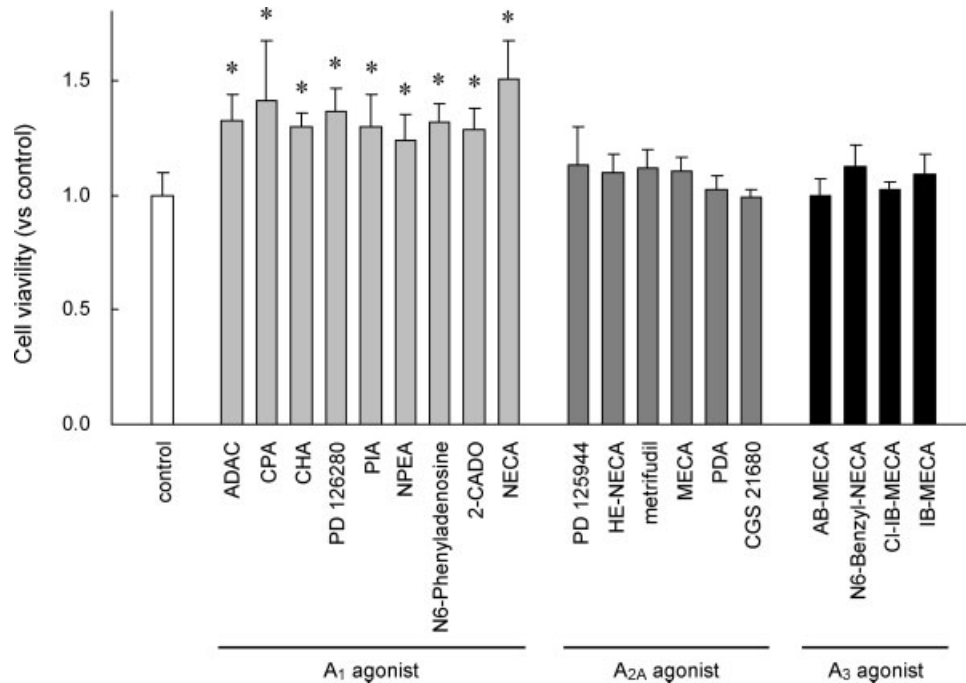


Fig. 5. Adenosine A₁ agonists induce proliferation of neural stem cells. NSCs were treated with the indicated A₁, A₂, and A₃ agonists (100 nM) or DMSO (control) for 3 days, and cell proliferation was measured. Results are presented as the magnitude of the increase relative to the control (* $P < 0.05$ vs. control).

A₁ agonist-induced proliferation of NSCs. CPA-induced cell proliferation was markedly inhibited (36% decrease) by U0126, an inhibitor of MEK kinase (Klingenberg et al., 2004), although U0126 decreased 18% of cell proliferation of the control (Fig. 7). API-2, which is an inhibitor of Akt (Yang et al., 2004), also attenuated CPA-induced NSC proliferation (38% decrease) and tended to inhibit cell proliferation of control (25% decrease). These results suggested that MEK and Akt might be essential kinases for NSC proliferation and, in particular, for adenosine A₁ agonist-induced cell proliferation.

DISCUSSION

In this study, we demonstrated that adenosine A₁ and A_{2B} receptors are dominantly expressed in NSCs and that adenosine A₁ receptor agonists induce proliferation of NSCs. In addition, we found that the A₁ agonist activated MEK, ERK, and Akt and that MEK and Akt inhibitors attenuated A₁ agonist-induced NSC proliferation. These observations suggest that activation of the adenosine A₁ receptor stimulated NSC proliferation via the MEK/ERK and Akt signaling pathways.

Adenosine is a naturally occurring nucleoside that is released in large quantities at a variety of pathological brain sites in Parkinson's disease, multiple sclerosis, and brain injury, such as ischemia, trauma, and epilepsy (Clark et al., 1997; Lynch et al., 1998; Weigand et al., 1999; Laghi et al., 2000; Robertson et al., 2001). Aden-

osine has many functions in the CNS that involve inhibition of neurotransmission and neuroprotective actions in pathological conditions. Adenosine's neuroprotective influences are thought to be mediated through its interaction with the adenosine A₁ receptor (Brundege and Dunwiddie, 1997; Schubert et al., 1997; Haas and Selbach, 2000). In addition, it is a key mediator of vasodilation in vascular beds, in which A₁ and A₂ receptors are likely to be involved. Therefore, adenosine and the involved receptors, particularly the adenosine A₁ receptor, play a functional role during acute CNS insults. In this study, the adenosine A₁ receptor was dominantly expressed in NSCs isolated from the SVZ of mouse brain (Fig. 1), and adenosine induced proliferation of NSCs (Fig. 2). Consistent with this result, activation of the adenosine A₁ receptor by various A₁ agonists was also shown to induce NSC proliferation (Fig. 5). Therefore, our results suggest that the adenosine A₁ receptor might influence NSC renewal in CNS pathologies.

NSCs are multipotent and self-renewing progenitor cells that can differentiate into neurons and glial cells. Therefore, they are expected to be of use in the treatment of neurodegenerative disorders such as Parkinson disease, multiple sclerosis, nerve injury, and stroke (Lois and Alvarez-Buylla, 1993; Morshead et al., 1998; Johansson et al., 1999). Many studies have been performed to understand the extrinsic factors involved in the proliferation and differentiation of NSCs and also to investigate the mechanisms regarding their signaling

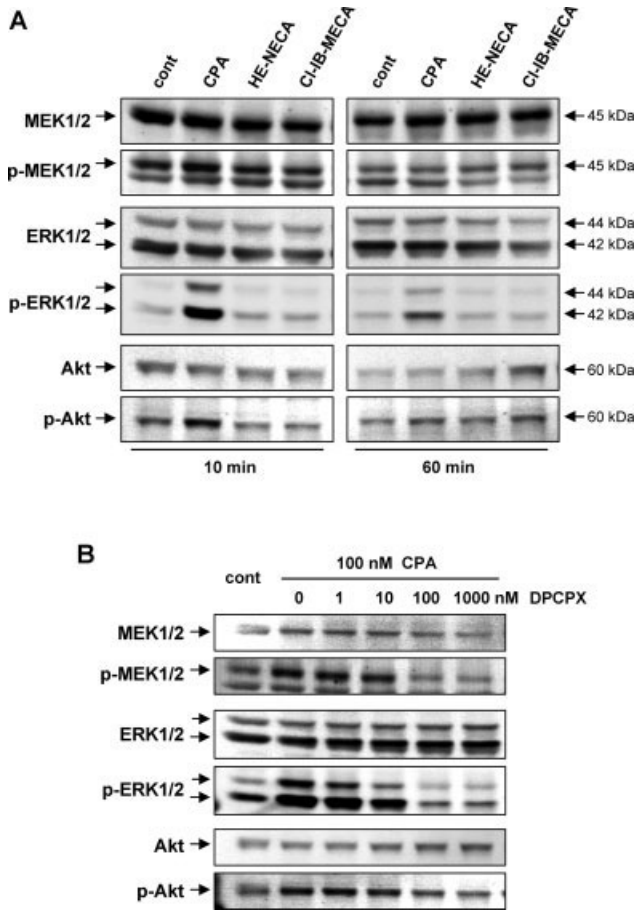


Fig. 6. CPA induces phosphorylation of MEK, ERK, and Akt. **A:** NSCs were treated with CPA (100 nM), HE-NECA (100 nM), CLIB-MECA (100 nM), or DMSO (control) for 10 or 60 min. **B:** NSCs were pretreated with DPCPX (0, 10, 100, or 1000 nM) for 30 min and then incubated with 100 nM CPA for 10 min. Western blot analysis was then performed.

pathways (Otaegi et al., 2006; Hagg, 2007; Kalluri et al., 2007; Kunath et al., 2007). bFGF plays a key role in regulating proliferation and maintenance of NSCs, which are in part mediated by MEK/ERK signaling pathways (Kunath et al., 2007). Our results indicated that 3-day incubation of NSCs with the MEK inhibitor U0126 decreased 18% of BrdU incorporation (control group; Fig. 7) and that BrdU incorporation of NSCs on day 3 increased 2.1-fold compared with that on day 0 (Fig. 2A). These results suggest that the MEK inhibitor does not induce the cell death but instead reduces basal cell proliferation. Therefore, consistent with previous observations (Kunath et al., 2007), our results suggest that MEK/ERK signaling pathways may be necessary for the proliferation of NSCs. In addition, our results indicate that the A₁ agonist CPA induces NSC proliferation (Fig. 2) and activates MEK/ERK (Fig. 6) and that the MEK inhibitor attenuates the induction of NSC proliferation by A₁ receptor activation (Fig. 7). Therefore, adenosine A₁ agonist-induced proliferation of NSCs

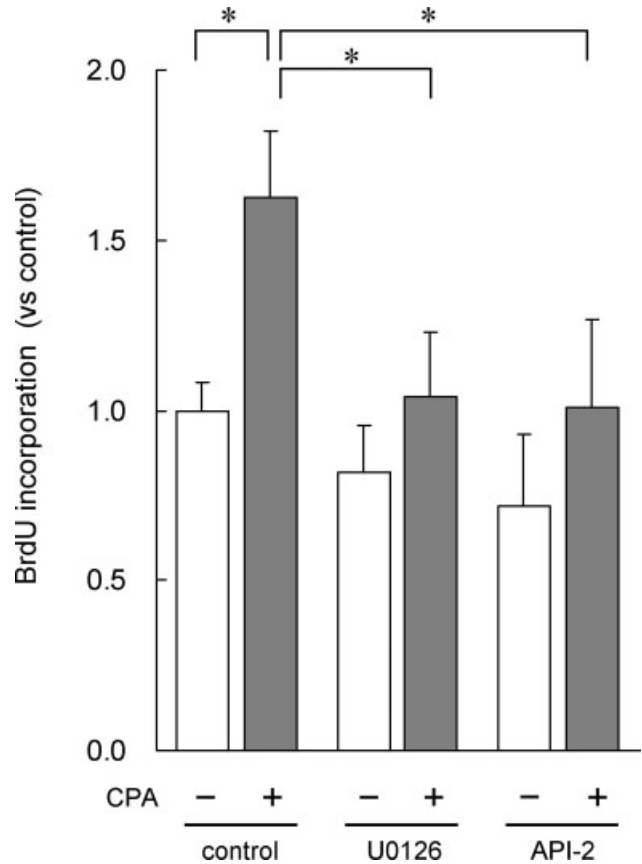


Fig. 7. CPA-induced proliferation of neural stem cells is attenuated by MEK and Akt inhibitors. NSCs were incubated with MEK inhibitor U0126 (100 nM) or Akt inhibitor API-2 (100 nM) in the presence or absence of CPA (100 nM) for 3 days prior to measurement of BrdU incorporation. Results are presented as the magnitude of the increase relative to the control without CPA (**P* < 0.05 vs. control without CPA).

may be mediated in part by MEK/ERK signaling pathways, which might interact with the signaling of other endogenous regulators such as bFGF. In addition to bFGF, insulin-like growth factor-I (IGF-I) is known to stimulate the proliferation of NSCs by activating ERK and phosphoinositide 3-kinase (PI3K), which then activate Akt. Akt is a key intrinsic factor for the proliferation and differentiation of stem cells including NSCs (Otaegi et al., 2006; Kalluri et al., 2007). The results in this report also demonstrate that the A₁ agonist CPA activates Akt (Fig. 6) and that the Akt inhibitor API-2 decreases CPA-induced cell proliferation as well as its basal cell proliferation (Fig. 7). Therefore, it is suggested that the proliferation of NSCs is induced by activation of the adenosine A₁ receptor and that its induction is mediated in part by Akt signaling pathways.

The Akt signaling pathway is important not only for cell proliferation but also for differentiation of NSCs (Otaegi et al., 2006; Kalluri et al., 2007). Therefore, we tested whether adenosine agonists induced differentiation

of NSCs. Agonists of A₁ (CPA), A_{2A} (HE-NECA), and A₃ (Cl-IB-MECA) did not show any significant effect on the expression of the stem cell marker nestin and various differentiation markers, such as the neuron marker β -tubulin III, the astrocyte marker glial fibrillary acidic protein, the oligodendrocyte marker cyclic nucleotide phosphodiesterase, and the oligodendrocyte marker myelin basic protein (Supp. Info. Fig. 1). Therefore, it is suggested that adenosine-mediated signals might not affect the differentiation of NSCs into neurons, astrocytes, and oligodendrocytes. Further studies are needed to verify this suggestion.

Adenosine is formed intracellularly in neural cells and then released by diffusion and transporters. It is also extracellularly generated from AMP by adenosine kinase. The basal adenosine level in the adult CNS is regulated in the range of 50–200 nM. Physiological concentrations are close to the binding affinity of the adenosine A₁ receptor for adenosine ($K_i = 300$ nM; Merighi et al., 2001), although there may be a discrepancy between physiological conditions and the *in vitro* situation. Thus, a small increase in adenosine concentration may affect adenosine A₁ receptor-mediated functions in the CNS including NSCs, which dominantly express the adenosine A₁ receptor (Fig. 1). A recent study found that adenosine level in hippocampal slices can increase to more than 20 μ M within 10 min of ischemia (Pearson et al., 2006). In this study, we observed that submicromolar levels of adenosine did not affect the proliferation of NSCs, whereas adenosine levels of 3–30 μ M stimulated proliferation of NSCs (Fig. 2). Therefore, we suggest that NSC proliferation might be induced by pathological conditions of the CNS such as ischemia, which enhance extracellular adenosine concentrations.

NSCs express the adenosine A_{2B} receptor (Fig. 1). We observed that the effective range of adenosine for stimulating proliferation of NSCs is 3–30 μ M (Fig. 2), which is similar to the range of functional potency (cyclic AMP assay) of the adenosine A_{2B} receptor for adenosine ($EC_{50} = 20$ –30 μ M; Merighi et al., 2001). Therefore, adenosine may affect NSCs via the adenosine A_{2B} receptor. NECA is an A₁ agonist that has been used as a nonselective agonist. It recently has been reported that NECA increases cyclic AMP levels and activates ERK in adenosine A_{2B} receptor-overexpressed Chinese hamster ovary cells (Schulte and Fredholm, 2003). However, the physiological role of adenosine A_{2B} receptor-mediated MAPK phosphorylation is still unclear. In this study, we observed that NECA significantly induces proliferation of NSCs; however, we believe that the effect may be mediated through the adenosine A₁ receptor because the expression of the adenosine A₁ receptor in NSCs and the affinity of the adenosine A₁ receptor for adenosine were higher than those of the adenosine A_{2B} receptor.

Mishra and colleagues (2006) recently reported P2 receptor-mediated NSC proliferation; ATP, ADP, and UTP induce NSC proliferation. In addition, they demonstrated that 1 μ M of adenosine augments NSC prolif-

eration. In the present study, NSC proliferation was observed at 10 and 30 μ M of adenosine. We demonstrated NSC proliferation in a monolayer, and we also tested it in neurospheres, which were used by Mishra et al. (2006). Adenosine A₁ receptor agonist CPA only induced proliferation of NSCs in the monolayer and the neurospheres, but the A₂ agonist HE-NECA and the A₃ agonist Cl-IB-MECA did not affect it (Supp. Info. Fig. 2). Differences in assay conditions did not cause the discrepancy between their results and those of the present study. The discrepancy might be a result of adenosine being added daily to NSCs during 3 days of culture in the Mishra et al. study, and therefore, they might have observed adenosine-induced cell proliferation at lower concentration rather than the concentration used in the present study. Furthermore, Mishra et al. indicated that high concentrations (10 and 50 μ M) of adenosine reduced the cell number dramatically. We also found that 100 and 300 μ M adenosine did not induce NSC proliferation (Supp. Info. Fig. 3A), although it might reflect physiological and pathological concentrations. The high concentrations of adenosine did not show cell toxicity (Supp. Info. Fig. 3B), and therefore, we have not identified the mechanisms by which adenosine stimulates cell proliferation. There is a possibility that high concentrations of adenosine may activate the adenosine A_{2B} receptor, which signals via G_s protein and has the opposite effect of adenosine A₁ receptor-coupled G_i protein.

In conclusion, we have demonstrated that adenosine and adenosine A₁ agonists stimulate the proliferation of NSCs, which dominantly express adenosine A₁ and A_{2B} receptors. We have further characterized A₁ agonist-induced cell proliferation and shown that A₁ agonists induce the phosphorylation of MEK, ERK, and Akt and that the inhibitors of MEK and Akt attenuate A₁ agonist-induced cell proliferation. These findings show that activation of the adenosine A₁ receptor stimulates proliferation of NSCs via the MEK/ERK and Akt signaling pathways; this suggests that the adenosine A₁ receptor may be a target molecule for regulating neuroregeneration.

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