Essential Role of CREB Activation by A$_{2A}$ Adenosine Receptors in Rescuing the NGF-induced Neurite Outgrowth Impaired by Blockage of the MAPK Cascade

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Running title: Neuronal Differentiation and A$_{2A}$ Adenosine Receptors
Summary

We found in the present study that stimulation of the A2A adenosine receptor (A2A-R) using an A2A-selective agonist (CGS21680) rescued the blockage of NGF-induced neurite outgrowth when the NGF-evoked MAPK cascade was suppressed by an MEK inhibitor (PD98059) or by a dominant-negative MAPK mutant (dnMAPK). This action of A2A-R (designated as the A2A-rescue effect) can be blocked by two inhibitors of protein kinase A (PKA), and was absent in a PKA-deficient PC12 variant. Activation of the cAMP/PKA pathway by forskolin exerted the same effect as that by A2A-R stimulation. PKA thus appears to mediate the A2A-rescue effect. Results from CREB phosphorylation at serine\(^{133}\), trans-reporting assays, and over-expression of two dominant-negative CREB mutants revealed that A2A-R stimulation led to activation of CREB in a PKA-dependent manner and subsequently reversed the damage of NGF-evoked neurite outgrowth by PD98059 or dnMAPK. Expression of an active mutant of CREB readily rescued the NGF-induced neurite outgrowth impaired by dnMAPK, further strengthening the importance of CREB in the NGF-mediated neurite outgrowth process. Moreover, simultaneous activation of the A2A-R/PKA/CREB-mediated and the PI3K pathways caused neurite outgrowth which was not suppressed by a selective inhibitor of TrkA, indicating that transactivation of TrkA was not involved. Collectively, CREB functions in conjunction with the PI3K pathway to mediate the neurite outgrowth process in PC12 cells.

Key words: A\(_2\)A adenosine receptor, nerve growth factor, CREB, neurite outgrowth, PI3K, PKA, MAPK.
Introduction

Adenosine has been shown to play an essential role in modulating neuronal function via adenosine receptors (1). To date, four adenosine receptors (A1, A2A, A2B, and A3) have been cloned and characterized (2). In the central nervous system, the A2A-R gene is heavily expressed by striatal neurons and colocalizes with the D2 dopamine receptor in GABAergic striopallidal neurons (3). In addition, A2A-R was found in cholinergic striatal neurons (4). Evidence from several laboratories suggests that A2A-R is involved in the regulation of synaptic plasticity (5) and may play a critical role in early neuronal development (6).

Involvement of neurotrophic factors in regulating neuronal development has been well established (7, 8). In the striatum, nerve growth factor (NGF) is expressed in GABAergic neurons (9); its receptors (TrkA and p75) are located in cholinergic interneurons, which also express A2A-R (4, 10). Colocalization of NGF receptors and A2A-R in the striatum suggests a potential cross-interaction between the signaling pathways evoked by NGF and adenosine. Because PC12 cells express both A2A-R and NGF receptors (11, 12), these cells were chosen in the present study to investigate the potential function of A2A-R in regulating NGF’s function. PC12 cells have been widely used to delineate the molecular mechanisms evoked by NGF because they differentiate into sympathetic neuronal-like cells upon NGF treatment and acquire numerous neuronal characters (11). NGF has been shown to activate multiple signaling pathways in PC12 cells (13, 14). Some of these pathways (e.g., PI3K/Akt, p53) appear to mostly mediate the anti-proliferative effect of NGF, while others (e.g., the Ras/Raf/MEK/MAPK and the PI3K/Rac pathways) have been mainly implicated in the differentiation process (15-17). Integration of multiple signaling pathways activated by NGF might be required to trigger the neuronal differentiation process since sustained activation of either the MAPK or the PI3K pathway by itself is insufficient to induce neurite outgrowth in PC12 cells (18, 19). Such complexity in multiple signaling pathways evoked by NGF permits a highly regulated signal cascade to trigger coordinated cellular responses.
We have previously demonstrated in PC12 cells that stimulation of A2A-R activates at least two major cellular signaling cascades transduced by adenylyl cyclase/protein kinase A (PKA) and protein kinase C (PKC) (20-23). Activation of both PKA- and PKC-mediated pathways by A2A-R was also observed in striatal cholinergic neurons (24). In the present study, we demonstrate that activation of A2A-R potentiates NGF-induced neurite outgrowth, especially at sub-maximal concentrations of NGF. More strikingly, A2A-R stimulation rescues the ability of PC12 cells to proceed with NGF-evoked neurite outgrowth when the MAPK cascade is abated (designated as the A2A-rescue effect). Pharmacological and molecular biological analyses suggest that PKA-stimulated activation of CREB mediates the A2A-rescue effect. Although necessary, activation of CREB is not sufficient to trigger the neurite outgrowth process. Instead, simultaneous activation of the CREB- and the PI3K-mediated pathways induced neurite outgrowth in PC12 cells, indicating that these two pathways function in coordination to trigger the neuronal outgrowth process.

**Experimental procedures**

**Reagents:** All reagents were purchased from Sigma Chemical (St. Louis, MO) except where specified. Forskolin, 2-p-(2-carboxyethyl) phenethylamino-5’-N-ethylcarboxyamidoadenosiner (CGS 21680; CGS), and 8-(3-chlorostyryl) caffeine (CSC) were purchased from Research Biochemical (Natick, MA). DMEM, fetal bovine serum, and horse serum were purchased from Life Technologies (Gaithersburg, MD). H-89 was from Biomol (Plymouth Meeting, PA). Anti-MAPK antibodies and Tfx™ were purchased from Promega (Madison, WI). The anti-active CREB antibody was obtained from Santa Cruz Biotech (Santa Cruz, CA). The anti-CREB antibody was a generous gift from Dr. M-J Lai (Institute of Molecular Biology, Academia Sinica, Taiwan). NGF was obtained from Alomone (Jerusalem, Israel). The anti-HA antibody was purchased from Roche Applied Science (Mannheim, Germany). The Phospho-Akt (Ser473) antibody and the Akt antibody were purchased from Cell Signaling Technology (Beverly, MA).
Cell Culture: PC12 cells were originally obtained from ATCC (CRL1721) and maintained in DMEM (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Gibco) plus 10% horse serum (Gibco) in an incubation chamber gassed with 10% CO₂-90% air at 37 °C. A123, a cAMP-dependent protein kinase (PKA)-deficient variant of PC12 cells (25), was kindly provided by Dr. J.A. Wagner (Cornell University Medical College, USA). A123 cells were maintained in DMEM supplemented with 5% v/v horse serum and 10% v/v fetal bovine serum. Cells were grown on tissue-culture plates coated with poly-L-lysine (Sigma). To trigger neuronal differentiation, cells were treated with NGF of the indicated concentration in the growth medium for 4 days. Cells containing neurites of at least 2 cell-body diameters in length were scored as neurite-bearing cells.

Transfection and neuronal differentiation: All plasmids used in the transient transfection experiments were prepared by CsCl gradient purification. The pCMV-p41(Ala₅₄Ala₅₅)mapk (dnMAPK) plasmid encodes a kinase-dead erk2 (MAPK) mutant (26). The expression constructs, which encode a dominant-negative S133A-CREB mutant (CREBm1), a dominant-negative CREBR287L mutant, a protein kinase inhibitor (PKI), a dominant-negative mutant of PI3K (Δp85), an HA-Akt, and a constitutively active PI3K mutant (p110*), are described elsewhere (27-33). CREB-VP16 was constructed by subcloning a Bam HI/Bam HI fragment encoding the full-length CREB into the corresponding sites of a pVP16 vector to produce a fusion protein of the VP16 activation domain to the full-length CREB. Cells were transfected using Tfx™ (Promega) following the manufacturer's protocol. Transfection efficiency was typically between 10% and 15%. For analyzing neuronal differentiation, cells were plated at 1 x 10⁵ cells per 35-mm dish and transiently transfected with the indicated construct(s) along with 1/15 of the molar amount of an expression construct (pEGFP, Clontech; Palo Alto, CA) encoding a green fluorescent protein (GFP). After transfection, cells were treated with the indicated reagent(s) for 3 days. Transfected cells were marked as GFP-expressing cells under a fluorescent microscope with a blue filter. Cells containing neurites of at least 2 cell-body diameters in length were scored as neurite-bearing cells. Transfected cells that grew neurites were normalized to the number of total transfected cells and are presented as the percentage of neurite-bearing cells. For each transfectant, at least 100 transfected
cells were counted. Data points represent the mean ± SEM of at least 3 independent experiments.

**Luciferase assay:** The function of dnMAPK and the activation of CREB were determined using the Elk1 and the CREB Trans-Reporting System (Stratagen, Seattle, WA), respectively, following the manufacturer’s protocol. To determine CREB activation, the expression vector encoding the Gal4-DNA binding domain (dbd) or chimeric Gal4dbd-CREB protein was co-transfected with a pRL-SV40 plasmid and a pFR-Luc reporter plasmid into PC12 cells. Twenty-four hours post transfection, cells were treated with indicated reagent(s) for 3 h and harvested for luciferase assay. To examine the effect of dnMAPK, PC12 cells were transfected with a pRL-SV40 plasmid, a pFR-Luc reporter plasmid, a Mek1 constitutively active mutant pFC-Mek1 (34), and an empty vector or the dnMAPK encoding vector, for 48 h. Cells were then harvested for luciferase assay. The pRL-SV40 plasmid encodes a reporter gene (*Renilla* luciferase, R-LUC) driven by the SV40 promoter and serves as an internal control. The pFR-Luc reporter plasmid contains a synthetic promoter with 5 tandem repeats of the yeast GAL4 binding sites which control the expression of the firefly luciferase gene (F-LUC). Activities of F-LUC were normalized to those of R-LUC and were utilized to determine the level of CREB activation or the effect of dnMAPK. To determine the dominant negative effect of 2 CREB mutants (CREBm1 and CREBR287L), a CRE reporter construct was utilized. The CRE reporter plasmid, generously provided by Dr. J. J.Y. Yen (IBMS, Academic Sinica, Taiwan), contains a synthetic promoter with 3 tandem repeats of CRE which regulate the expression of the F-LUC gene. The expression vector encoding CREBm1 or the CREBR287L protein was co-transfected with the pRL-SV40 plasmid and the CRE reporter plasmid into PC12 cells. Twenty-four hours post transfection, cells were treated with indicated reagent(s) for 3 h and harvested for luciferase assay. Activities of F-LUC were normalized to those of R-LUC.

**Immunoprecipitation:** Cells were disrupted by addition of a lysis buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin A, 1 mM Na3VO4, 1 mM dithiothreitol (DTT), and 100 nM okadaic acid.
Lysates were passed 5 times through a 25-gauge syringe and then incubated for 15 min at 4 °C under constant rotation. Debris and unbroken cells were removed by centrifugation. The anti-HA antibody (3 µg) was first purified using Sephadex-conjugated protein A (Sigma), and then washed twice with ice-cold phosphate-buffered saline (PBS) and once with the lysis buffer (4 °C). HA-Akt was immunoprecipitated by incubating the total lysates with anti-HA–Sephadex-conjugated protein A beads at 4 °C with constant rotation for 17 h. The HA-Akt immunocomplexes were washed 4 times with the washing buffer (25 mM HEPES [pH 7.8], 10% [v/v] glycerol, 1% [v/v] Triton X-100, 0.1% [wt/v] bovine serum albumin [BSA], 0.5 M NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 100 nM okadaic acid), and then boiled in the presence of 2X SDS sample treatment buffer for subsequent separation by SDS-PAGE followed by Western blot analyses.

**Western blot analysis:** Protein concentrations were determined using the Bio-Rad Protein Assay Dye Reagent. Equal amounts of sample were separated by SDS-PAGE using 10% polyacrylamide gels (35). The resolved proteins were then electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with 1% bovine serum albumin, and incubated with the desired primary antibody at 4 °C overnight, followed by the corresponding secondary antibody for 1 h at room temperature. Typically, we used a 1: 2000 dilution for both anti-CREB and anti-phosphorylated CREB antibodies, a 1: 1000 dilution for both anti-MAPK and anti-phosphorylated MAPK antibodies, and a 1: 1000 dilution for both anti-Akt and anti-phosphorylated Akt antibodies. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) and recorded using Kodak XAR-5 film.
Results

Stimulation of A2A-R modulated NGF-induced neurite outgrowth in PC12 cells.

By using neurite outgrowth as a marker of differentiation, the effect of A2A-R stimulation in modulating NGF-induced neuronal differentiation in PC12 cells was examined. As shown in Fig. 1A, stimulation of A2A-R using an A2A-selective agonist (CGS21680; CGS) enhanced the percentage of neurite-bearing cells at sub-maximal doses of NGF. At high doses of NGF (> 50 ng/ml), the effect of CGS on NGF-evoked neurite outgrowth was not significant.

Accumulating evidence suggests that NGF evokes neuronal differentiation in PC12 cells through multiple processes (16, 36). Among these NGF-evoked signals, the Ras/Raf/MEK/MAPK kinase cascade (designated as the MAPK cascade) was shown to be required for NGF-induced differentiation in PC12 cells (16). Since the MAPK-mediated pathway can be inhibited under a number of cellular conditions (e.g., oxidative stress; 37), we next determined the effect of A2A-R stimulation on NGF-induced neurite outgrowth when the MAPK cascade was suppressed by a MEK-specific inhibitor (PD98059). As shown in Fig. 1B, PD98059 markedly suppressed NGF-induced neurite outgrowth as reported elsewhere (16), demonstrating that the MAPK-mediated pathway is critical for NGF-induced neurite outgrowth. Most strikingly, stimulation of A2A-R using CGS rescued the blockage of neurite outgrowth by PD98059 (Fig. 1B). We designated this rescuing effect of A2A-R on the suppression of NGF-induced neurite outgrowth due to blockage of the MAPK cascade the “A2A-rescue effect”.

Others and ourselves have previously reported that stimulation of A2A-R leads to activation of the ERK/MAPK pathway in PC12 cells (23, 38). It is then of great interest to determine whether the A2A-rescue effect is mediated by activation of MAPK. As illustrated in Fig. 2, treatment with NGF increased phosphorylation of MAPK without altering protein levels. Stimulation of A2-R by CGS also slightly enhanced phosphorylation of MAPK when a long exposure time was used for the autoradiography of the Western blot analysis (data not shown) as previously reported (23). However, the
level of increased MAPK phosphorylation by CGS was significantly lower than that by NGF and therefore could not be effectively detected with the short exposure time used for the autoradiography film shown in Fig. 2. Treatment with PD98059 blocked NGF-mediated activation of MAPK. Stimulation of A2A-R did not further enhance the phosphorylation of MAPK by NGF in either the absence or the presence of PD98059. The inhibition of NGF-evoked MAP kinases by PD98059 was not reversed by A2A-R stimulation, implying that the A2A-rescue effect on neurite outgrowth does not require MAPK activation.

To further establish that MAPK activation is not involved in the A2A-rescue effect, we further utilized a dominant-negative MAPK expression construct (dnMAPK; 26) for the experiments of suppressing NGF-evoked neurite outgrowth. The dominant negative effect of dnMAPK was first verified using a PathDetect trans-reporting system. As shown in Fig. 3A, expression of a constitutively active mutant of MEK1 (Mek1; 34) provided by the manufacturer (Stratagen) markedly enhanced the MAPK-specific, trans-acting activity of Elk1 in PC12 cells. Simultaneous expression of dnMAPK significantly reduced the Mek1-enhanced reporter activity. As predicted, expression of dnMAPK in PC12 cells inhibited NGF-evoked neurite outgrowth. Consistent with the above observation, stimulation of A2A-R using CGS rescued the blockage of neurite outgrowth by dnMAPK (Fig. 3B, C). Furthermore, pretreating cells with an A2A-R-selective antagonist, 8-(3-chlorostyryl) caffeine (CSC), markedly reduced the effect of CGS (Fig. 3C, D). Thus, the rescue effect of CGS is mediated specifically by A2A-R. These results suggest that stimulation of A2A-R triggers a signal which functions downstream of MAPK to rescue the blockage of NGF-induced neurite outgrowth by PD98059 or by dnMAPK.

PKA, but not PKC, mediates the A2A-rescue effect on NGF-induced neurite outgrowth in PC12 cells.

Since activation of A2A-R leads to a transient increase in cAMP in PC12 cells (12), we first examined whether PKA plays an important role in the A2A-rescue effect. As shown in Fig. 4, direct stimulation of adenylyl cyclase using forskolin (FK), which
subsequently activates the cAMP/PKA pathway, also rescued the blockage of neurite outgrowth by dnMAPK in PC12 cells. Treatment with a selective PKA inhibitor (H-89; Fig. 4A) or transient over-expression of a peptide inhibitor of PKA (PKI; Fig. 4B) blocked the rescue effect of CGS and FK. In addition, CGS exerted no rescue effect in a PKA-deficient PC12 variant (A123, Fig. 4C), further supporting our hypothesis that PKA is critical for the A2A-rescue effect. Because the cAMP-response element-binding protein (CREB) is a point of convergence for the cAMP/PKA and MAPK pathways (39, 40), we next examined whether stimulation of A2A-R led to activation of CREB via PKA. As shown in Fig. 5, stimulating PC12 cells with CGS increased the phosphorylation levels of CREB at Ser133. This CGS-evoked phosphorylation of CREB could be suppressed by a PKA-selective inhibitor (H89), suggesting a potential role of CREB in mediating PKA’s action and contributing to the A2A-rescue effect.

In contrast, a general inhibitor (chelerythrine, CHE) did not alter the A2A-rescue effect, indicating that PKCs are not involved in this action of A2A-R (Table 1). Consistent with the involvement of PKCs in NGF-induced neuronal differentiation reported elsewhere (41), treatment with CHE alone slightly reduced NGF-induced neurite outgrowth in our clone of PC12 cells (Table 1).

CREB functions downstream of MAPK to mediate the A2A-rescue effect.

To further examine the involvement of CREB in the A2A-rescue effect, we assessed the activation of CREB by determining its phosphorylation levels at Ser133 using Western blot analysis. As shown in Fig. 6, treating PC12 cells with CGS or NGF markedly increased CREB phosphorylation. Stimulation of A2A-R using CGS slightly enhanced NGF-evoked CREB phosphorylation. Treatment with PD98059 significantly suppressed phosphorylation of CREB by NGF, whereas that by A2A-R stimulation was much less affected (Fig. 6B). In the presence of PD98059, simultaneous activation of A2A-R and NGF receptors induced a significantly higher level of CREB phosphorylation than that by NGF alone. To demonstrate whether CREB was activated and if the CREB mediated gene expression was enhanced during the above conditions, we next employed
a CREB Trans-Reporting System to assess the activity of CREB. As shown in Fig. 6C, the activity of CREB was significantly enhanced by treatment with CGS and/or NGF. As predicted, NGF-evoked CREB activation was reduced by PD98059. Most importantly, in the presence of PD98059, the addition of CGS restored CREB activation when compared to treatment with NGF alone. Collectively, upon blockage of the MAPK cascades, A2A-R stimulation allowed PC12 cells to retain CREB activation to a level no less than that induced by NGF. This observation is consistent with our hypothesis that CREB activated by PKA might function downstream of MAPK to mediate the A2A-rescue effect in PC12 cells.

To verify the role of CREB in the A2A-rescue effect, we next transiently expressed 2 dominant negative CREB mutants (CREBm1 and CREB-R287L) in PC12 cells. The CREBm1 mutant (i.e., S133A-CREB) retains its ability to bind to the CRE site, but is incapable of being activated by phosphorylation at Ser\(^{133}\) that plays a critical role in its transcriptional activation (42). The other dominant negative mutant of CREB (CREBR287L) contains a mutation in the DNA binding domain of CREB (33). The dominant negative effect of CREBm1 and CREB-R287L was assessed using a CRE-reporter construct which contains a synthetic promoter with 3 tandem repeats of CRE to regulate the expression of the F-LUC reporter gene. As expected, stimulation of A2A-R using CGS markedly enhanced the expression of the reporter gene (Fig. 7A). Expression of CREBm1 or CREB-R287L significantly reduced the CRE-mediated reporter activity upon A2A-R stimulation, demonstrating the inhibitory effect of these 2 CREB mutants. Most importantly, expression of CREBm1 and CREB-R287L effectively inhibited NGF-induced neurite outgrowth and the A2A-rescue effect (Fig. 7B, C). Together, these results suggest that CREB plays a critical role in the A2A-rescue effect. To determine whether activation of the CREB-mediated signaling pathway is sufficient to elicit the A2A-rescue effect, a construct encoding a constitutively active form of CREB, the herpes virus VP16 transactivation domain fused to CREB (CREB-VP16; 43), was over-expressed in PC12 cells by transient transfection. As shown in Fig. 8, over-expression of CREB-VP16 rescued NGF-induced neurite outgrowth impaired by dnMAPK. Note that expression of CREB-VP16 in the absence of NGF induced no neurite outgrowth. Collectively,
activation of CREB activity is both necessary and sufficient for the A2A- rescue effect in PC12 cells. However, activation of the CREB by itself is not sufficient to trigger neurite outgrowth in PC12 cells.

*Simultaneous activation of the cAMP/CREB- and the PI3K pathways triggers the neurite outgrowth process.*

Previous studies have shown that sustained activation of MAPKs is insufficient to evoke differentiation in PC12 cells (18). Other downstream effectors of TrkA (such as PLCγ, p38, and PI3K) have also been implicated in NGF-mediated neuronal differentiation in PC12 cells (17, 18, 44). Since activation of the PKA/CREB pathway by itself did not cause neurite outgrowth as described above, we set out to identify the signaling pathway, which functions in conjunction with CREB to trigger the neurite outgrowth process. Because PI3K has been reported to play a critical role in NGF-induced neuronal differentiation, we next examined the function of PI3K by over-expressing a dominant negative mutant of the regulatory subunit of PI3K (Δp85; 27). This mutant lacks the iSH2 region, which is responsible for its interaction with the catalytic subunit of PI3K (p110), but retains the ability to associate with proteins containing phosphorylated tyrosine residues. Expression of Δp85 can compete with endogenous p85 proteins recruited by activated TrkA receptors and subsequently prevent the activation of p110 (27). To evaluate the inhibitory effect of Δp85, a construct encoding an HA-tagged wild-type Akt (a downstream target of PI3K; 45) was cotransfected with the expression construct of Δp85 in PC12 cells in the presence or absence of NGF as indicated. The transfected HA-Akt was immunoprecipitated and analyzed for its phosphorylation at Ser473 as an indication of PI3K activation. Exposure to NGF for 15 min significantly enhanced the phosphorylation (activation) of Akt. Such NGF-evoked Akt phosphorylation was dramatically reduced by the expression of Δp85 (Fig. 9A). As shown in Fig. 9B, C, expression of Δp85 significantly suppressed NGF-evoked neurite outgrowth. In addition, the suppressing effect of Δp85 could not be rescued by stimulation of A2A-R. Nor could A2A-R stimulation restore NGF-induced neurite outgrowth when both the MAPK cascade and the PI3K pathway induced by NGF
were blocked. Simultaneous activation of the PI3K pathway by NGF therefore appears to be important for the A2A-rescue effect.

To confirm the involvement of the PI3K-mediated pathway in the neurite outgrowth process, we transiently expressed a constitutively active mutant of p110 (p110*) in PC12 cells. Forty-eight hours post transfection, expression of p110* protein was readily detected by Western blot analysis (Fig. 10A). A marked increase in the phosphorylation (activation) of Akt was also observed, demonstrating the increase in PI3K activity (Fig. 9B). However, no significant neurite outgrowth in control (non-treated) PC12 cells was observed. In contrast, expression of p110* in cells treated with cAMP-elevating reagents (CGS or forskolin) induced neurite outgrowth (Fig. 10C, D). Such induction of neurite outgrowth by cAMP and p110* could be blocked by a dominant-negative mutant of CREB (CREB-R287L), but not by a dominant-mutant of MAPK (dnMAPK, Fig. 10E). Moreover, simultaneous expression of an active CREB mutant (CREB-VP16) and p110* caused neurite outgrowth (Fig. 10F). Collectively, these findings support our hypothesis that CREB functions downstream of MARK and cooperates with the PI3K-mediated pathway to induce the neurite outgrowth process.

An interesting study recently reported that TrkA receptors can be activated through stimulation of A2A-R in a PC12 variant over-expressing high levels of Trk A receptors (46). Although unlikely, it is possible that, in parental PC12 cells, A2A-R stimulation might also cause activation of a Trk-mediated pathway, and subsequently lead to neurite outgrowth of PC12 cells. To further address this possibility, a Trk-selective inhibitor (K252a; 47) was employed in the experiments where PC12 cells were treated with the combination of CGS treatment and overexpression of p110*. At the concentration (100 nM) utilized in the present study, K252a completely suppressed the NGF (100 ng/ml)-induced neurite outgrowth (24.1% ± 1.7% and 0.2% ± 0.2% in the absence and presence of K252a, respectively; 3 independent experiments) in PC12 cells. In contrast, addition of K252a at the same concentration did not alter neurite outgrowth induced by concurrent stimulation of PI3K and the A2A-R-induced signaling pathway (Fig. 11). Thus, the potential cross-talk between A2A-R and TrkA appeared not to contribute to the neurite outgrowth evoked by A2A-R stimulation and PI3K activation.
Discussion

We demonstrate in the present study that stimulation of A2A-R significantly enhances NGF-induced neurite outgrowth at sub-optimal concentrations of NGF, and rescues NGF-induced neurite outgrowth impaired by blockage of the MAPK cascade. This rescue effect of A2A-R requires PKA activation, since it is blocked by 2 different PKA inhibitors (H-89 and PKI), and is absent from a PKA-deficient PC12 variant (Fig. 4). In addition, activating the cAMP/PKA pathway by forskolin also rescues NGF-induced neurite outgrowth impaired by dnMAPK (Fig. 4). Although MAPK is activated by stimulation of A2A-R, MAPK activation is not responsible for the A2A-rescue effect. PKC was also uninvolved in the A2A-rescue effect because a general PKC inhibitor did not alter this action of A2A-R (Table 1). Based on the following evidence, we further conclude that CREB serves as a key mediator which functions downstream of PKA to elicit the A2A-rescue effect. First, stimulation of A2A-R led to activation of CREB which could be blocked by a PKA inhibitor (Fig. 5). Second, A2A-R retained its ability to activate CREB in the presence of PD98059 (Fig. 6). Third, activation of CREB was critical for the A2A-rescue effect since A2A-R’s action was abated by over-expression of 2 dominant negative CREB mutants (CREBm1 or CREB-R287L; Fig. 7). Finally, over-expression of a dominant positive CREB was sufficient to rescue NGF-induced neurite outgrowth damaged by dnMAPK (Fig. 8). CREB therefore appears to be a point of convergence for the MAPK cascade and the cAMP/PKA pathway, and mediates the A2A-rescue effect. Since activation of CREB alone by various means is insufficient to induce neurite outgrowth (Figs. 1,8), another signal evoked by NGF in addition to CREB is required for inducing neurite outgrowth. Using a dominant negative and a constitutively active mutant of PI3K (Δp85 and p110*, respectively) and an active mutant of CREB (CREB-VP16), we found that simultaneous activation of the CREB-signaling pathway and the PI3K-mediated pathway, another well-characterized pathway activated by NGF, is sufficient to induce neurite outgrowth (Figs. 9, 10). Since this enhancing effect by A2A-R stimulation and p110* was insensitive to the Trk-selective inhibitor, K252a (Fig. 11), cross-talk between A2A-R and TrkA did not play a role in the above observation.
Collectively, we demonstrate in the present study that concurrent activation of the CREB- and the PI3K-mediated pathways is sufficient to trigger neurite outgrowth in PC12 cells (Fig. 2).

We have previously reported that stimulation of \textit{A}_{2\text{A}}\text{-R} leads to an elevation of intracellular cAMP and activation of PKA which mediate the anti-apoptotic effect in PC12 cells (22, 23). Here, our data demonstrate that CREB functions downstream of PKA upon \textit{A}_{2\text{A}}\text{-R} stimulation to rescue the NGF-evoked neurite outgrowth impaired by blockage of the MAPK cascade. In addition to multiple experiments to demonstrate the necessity of PKA (Fig. 4A-C) and CREB (Figs. 6-8), exposure of PC12 cells to H89 blocked the CREB phosphorylation (activation) by \textit{A}_{2\text{A}}\text{-R} (Fig. 5). H89 is a selective PKA inhibitor which inhibits PKA in a competitive manner with ATP, and has been used to block PKA-mediated responses in many different cells including PC12 cells (48) at the concentration (10 \(\mu\text{M}\)) utilized in the present study. Similar to our findings, H89 at 10 \(\mu\text{M}\) significantly (but not completely) blocked cAMP-evoked protein phosphorylation and neurite outgrowth in PC12 cells (48). The incomplete blockage of the cAMP/PKA-evoked (48) or \textit{A}_{2\text{A}}\text{-R}-mediated (Fig. 5 of the present study) responses by H89 at 10 \(\mu\text{M}\) might have resulted from its poor membrane-penetrating ability or competition from intracellular endogenous ATP as suggested for another H series PKA inhibitor (H8, 49). Although H89 also slightly suppressed other kinases, the Ki values for other kinases are at least 10-fold (e.g., cGMP-dependent kinase, PKG) to approximately 3000-fold (e.g., casein kinase II) higher than those for inhibiting PKA. At 10 \(\mu\text{M}\), for which less than 40\% of the cAMP/PKA-mediated responses was inhibited (48), the non-specific inhibitory effect of H89 toward other kinases was expected to be minimal. Indeed, when PC12 cells were treated with up to 30 \(\mu\text{M}\) of H89, no significant effects on activities of endogenous PKG, PKC, and \(\text{Ca}^{2+}\)/calmodulin-dependent protein kinases were observed (48). Although we cannot completely rule out the involvement of kinases other than PKA in mediating the activation of CREB by \textit{A}_{2\text{A}}\text{-R}, collective results strongly suggest that PKA plays a major and critical role in mediating the CREB-dependent \textit{A}_{2\text{A}}\text{-rescue effect (Figs. 5-8).}
Implication of CREB in NGF-induced neurite outgrowth observed in the present study is consistent with the well-documented concept that CREB plays an important role in various functions of neurotrophins (43, 50). Our finding is also consistent with a recent report in which over-expression of dominant negative mutants of CREB attenuated NGF-evoked neurite outgrowth in PC12 cells (51). Accumulating evidence suggests that CREB can be phosphorylated by several kinases including MAPK and PKA (52), and in turn activates a specific set of gene transcriptions through binding to the CRE element (53). Upon phosphorylation of Ser\textsuperscript{133}, CREB binds to its coactivator (CREB-binding protein), which then interacts with the basal transcription factors and RNA polymerase II, and initiates transcription from the CRE element (39). In the present study, 2 methods (the CREB Trans-Reporting System, and the phosphorylation analysis of CREB-Ser\textsuperscript{133}) were employed to determine CREB activity. Results from both assays indicate that A\textsubscript{2A}-R stimulation elevated the NGF-evoked CREB activity reduced by PD98059 (Fig. 6). These results support our hypothesis that CREB mediates the A\textsubscript{2A}-rescue effect and functions as a key transcription factor for neurite outgrowth. We were surprised to find that the patterns of CREB activity determined using these 2 methods were not completely correlated (Fig. 6). Although the phosphorylation levels of CREB at Ser\textsuperscript{133} by NGF and by CGS21680 were similar (Fig. 6B), CREB activity upon A\textsubscript{2A}-R stimulation measured by the CREB trans-reporting system was markedly higher than that by NGF (Fig. 6C). It is also surprising to find that PD98059 decreased the activation of CREB by A\textsubscript{2A}-R, yet phosphorylation of CREB at Ser\textsuperscript{133} was not affected (Fig. 6B, C). These discrepancies might be due to different sensitivities of these 2 assays. Alternatively, A\textsubscript{2A}-R stimulation might utilize additional machinery, in addition to phosphorylation of Ser\textsuperscript{133} of CREB, to enhance CREB activation. Moreover, it is possible that as long as CREB activity is above a critical threshold for neurite outgrowth, the level of CREB activity might not necessarily be correlated with the extent of neurite outgrowth.

In addition to CREB, several other transcription factors (e.g., Fra, JunD, CREM, and ATF; designated as CREB isobinders) also bind to the CRE consensus site (39). A recent study demonstrated that NGF triggers a long-term increase in activity of an AP1 complex (containing Fra-2 and JunD), which binds to both AP1 and CRE consensus sites in PC12 cells. Since CREB-related constructs employed in the present study (i.e.,
CREBm1, CREBR287L, and VP16-CREB) may also interfere with the binding of CREB isobinders to the CRE element or hijack the cellular CRE isobinders, the involvement of CREB isobinders cannot be completely ruled out. Nevertheless, phosphorylation of CREB at Ser\textsuperscript{133} does correlate with neurite outgrowth (Fig. 6), which strengthens our hypothesis that CREB plays a critical role in the neurite outgrowth process. The CRE consensus site is found in many genes (e.g., c-fos, NGF-inducible A gene, NGF-inducible B gene, and the light neurofilament, Bcl2) important for neuronal functions (43, 54-56). For example, expression of Bcl-2 was activated by NGF through a CREB-mediated mechanism, and has been implicated in the survival of sympathetic neurons (43). We have tested the role of Bcl-2 in neurite outgrowth by over-expression of wild-type Bcl-2 in NGF-differentiated PC12 cells. Because over-expression of Bcl2 failed to restore the blockage of NGF-induced neurite outgrowth by dnMAPK (data not shown), Bcl2 might not play a critical role in mediating the neurite outgrowth process. It remains to be determined which gene(s) trans-activated by CREB mediates the induction of neurite outgrowth in PC12 cells.

Rapid activation of PI3K in just minutes upon NGF stimulation has been reported in PC12 cells (57). It is well documented that products of PI3K (phosphatidylinositol-3,4-P\textsubscript{2} and phosphatidylinositol-3,4,5-P\textsubscript{3}) can bind to the pleckstrin homology domain of important signal molecules including several GEFs (e.g., Sos and Vav) of Rac and subsequently cause activation of Rac1 (58, 59). Rac is a small GTPase promptly activated by NGF, and has previously been implicated in modulating polarized outgrowth of actin cytoskeleton during neuronal differentiation (17, 60). Using a PI3K inhibitor (LY2940042), Yasui and colleagues (17) suggested that PI3K regulates an initial marked activation of Rac and its recruitment to the protrusion sites of neurite upon NGF stimulation. Indeed, we found that expression of a dominant negative mutant of Rac (RacN17) significantly suppressed neurite outgrowth evoked by p110\textsuperscript{*} and cAMP (data not shown). Rac1N17 contains a mutation at Thr\textsuperscript{17} and is believed to inhibit the endogenous Rac by reducing its interaction with GEFs in a competitive manner. Rac therefore might function as one of the downstream targets of PI3K and play an important role in the neurite outgrowth process induced by pPI3K and cAMP. This observation is consistent with previous studies which suggest that the PI3K/Rac pathway is one of the
early signals which mediate NGF-induced neuronal differentiation in PC12 cells (17, 61). Interestingly, inhibition of MAPK using PD98059 did not elicit a significant effect on the formation of neurite protrusion or the redistribution of Rac by NGF. The MAPK cascade therefore is not required for initiation of neurite outgrowth, but rather, plays an important role in the extension of neurites (17). We have shown here that upon NGF stimulation, CREB is one of the major factors which functions downstream of the MAPK cascade, and works together with the PI3K-mediated pathway to induce neurite outgrowth. Note that the maximal percentage (17.5% ± 2.7%, 3 independent experiments, Fig. 10) of neurite outgrowth induced by 110* plus cAMP is less than that obtained by NGF (Fig. 1). It appears that, in addition to the MAPK/CREB- and the PI3K-mediated pathways, other signal(s) evoked by NGF might facilitate the neurite outgrowth process. Also, in addition to Rac, other molecules downstream of PI3K might be required for neurite outgrowth because expression of a constitutively active Rac mutant (Rac1L61; 31) did not result in neurite outgrowth in the absence or presence of cAMP-elevating reagents (data not shown). This finding is in agreement with a previous study using another Rac active mutant (RacV12; 62).

In the central nervous system, expression of A2A-R is found in many areas of the brain (6, 63). Due to its dynamic expression during development, A2A-R stimulation has previously been implicated in neuronal differentiation (6). Using PC12 cells as a neuronal model, we earlier reported that stimulation of A2A-R protected PC12 cells from apoptosis in PKA- and PKC-dependent manners. The MAPK cascade and the PI3K/Akt pathway are not required for the protective effect of A2A-R in PC12 cells (23). An interesting report (46) recently described how, in a PC12 variant [PC12(615)] which expresses 20-fold more Trk A than that of the parental PC12 cell, A2A-R stimulation causes phosphorylation of NGF receptors (Trk A) and induces activation of Akt (a downstream target of PI3K). Due to the over-expression of TrkA, differentiation of PC12(615) upon NGF treatment was markedly accelerated (64). The exact molecular mechanism mediating the interaction between A2A-R and TrkA in PC12(615) is largely unclear. It appears that mobilization of intracellular calcium and tyrosine phosphorylation are required for this transactivation of TrkA by adenosine receptors (65). In the PC12 cells employed in the present study, we observed no elevation of intracellular free
calcium levels measured by Fura-2 (data not shown), nor activation of Akt upon A2A-R stimulation (23). Such a discrepancy might be due to the fact that various signaling pathways were altered in a TrkA-overexpressing cell line such as PC12(615) (64). Moreover, a selective inhibitor (K252a) of TrkA exerted no effect on neurite outgrowth induced by A2A-R stimulation together with the constitutive activation of PI3K (Fig. 11). Stimulation of A2A-R in parental PC12 cells, such as those utilized in the present study, therefore might promote neurite outgrowth mainly through PKA-CREB mechanisms, but not via activation of the receptors (Trk A) of NGF.

In the present study, we report that A2A-R stimulation significantly enhances NGF-induced neurite outgrowth at sub-optimal concentrations of NGF, implying that A2A-R might be important for neuronal development in vivo. Our finding is consistent with earlier studies, which reported that adenosine and cAMP increase the rate of NGF-evoked neurite outgrowth (66, 67). The enhancing effect of A2A-R stimulation is likely to be mediated by the PKA/CREB pathway since transient expression of a CREB gain-of-function mutant (Tyr134Phe) in PC12 cells resulted in a significant increase in neurite outgrowth in the presence of sub-optimal doses of NGF (40). We also demonstrate that simultaneous activation of the CREB and the PI3K pathways are sufficient to elicit neurite outgrowth in PC12 cells. Via a cAMP/PKA/CREB-dependent pathway, A2A-R activation effectively rescues impaired NGF-induced neurite outgrowth resulting from a specific blockage of the MAPK pathway. Suppression of the MAPK-mediated pathway has been reported under various conditions. For example, MAPK activity is inhibited by interleukin-6 in excited hippocampal neurons (68). In addition, modulation of cellular oxidative stress using N-acetyl-L-cysteine (NAC) uncouples signal transduction from Ras to the MAPK cascade upon NGF treatment in PC12 cells (37). Moreover, persistent viral infection has been shown to impair NGF-evoked nuclear translocation of MAPK and neurite outgrowth in PC12 cells (69). Since activation of the MAPK cascade is critical for neurite outgrowth (16), impairment of the MAPK cascade in neurons might cause neuronal degeneration. Results presented herein imply that neuronal trauma and/or degeneration caused by suppression of the MAPK cascade might be rescued by A2A-R stimulation. Since A2A-R coexists with a NGF receptor (TrkA) in adult striatal cholinergic neurons (70), adenosine might also regulate striatal functions via cross-talk
between signals evoked by A$_{2A}$-R and TrkA. Further knowledge regarding the molecular mechanisms underlying A$_{2A}$-R’s action may facilitate the clinical application of A$_{2A}$-R agonists in the treatment of neuronal degeneration associated with nervous system trauma and neurological disease.
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Footnotes

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1List of non-standard abbreviations: A2A-R, A2A adenosine receptor; CGS, CGS 21680; CSC, 8-(3-chlorostyryl) caffeine; GPCR, G protein-coupled receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; FK, forskolin; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase.
Figure Legends

Fig. 1. Stimulation of A2A-R modulates neurite outgrowth evoked by NGF in PC12 cells. (A) PC12 cells were treated with the indicated concentration of NGF in the absence (circles) or presence (squares) of CGS21680 (CGS; 1 µM) for 3 days. (B) PC12 cells were pretreated with 20 µM of PD98059 (PD) for 30 min prior to addition of the indicated concentration of NGF in combination with (squares) or without (circles) CGS (1 µM) for 3 days. Percentages of cells bearing neurites longer than 2 cell bodies were quantified. Data points represent the mean ± SEM values from 3 different fields. Totals of at least 300 cells were scored for each condition. Data points are the mean ± SEM values from 3 independent experiments. *p < 0.05 compared to the corresponding non-CGS treated sample (two-way analysis of variance).

Fig. 2. Effect of A2A-R stimulation on activation of MAPK by NGF. PC12 cells were pretreated with or without PD98059 (PD; 100 µM) for 30 min before the addition of CGS (1 µM) and/or NGF (100 ng/ml) as indicated for 10 min. Phosphorylated MAPK (P-MAPK, upper panel) and total MAPK protein (lower panel) in cell lysates were quantified by Western blot analysis. Data shown are representative of results from 3 independent experiments.

Fig. 3. Stimulation of A2A-R rescues the suppression of NGF-evoked neurite outgrowth resulting from over-expression of a dominant-negative MAPK mutant (dnMAPK). (A) Expression vectors encoding the Gal4-DNA binding domain (dbd) or chimeric Gal4dbd-Elk protein were cotransfected with an empty vector or a constitutively active mutant of Mek1, a control vector or a dnMAPK encoding vector, and a TK promoter-driven R-luciferase (as an internal control) into PC12 cells together with an F-luciferase reporter gene driven by 5 copies of the Gal4-binding element. Forty-eight hours after transfection, cells were lysed and assayed for luciferase activity. The activity of F-luciferase was normalized to the activity of R-luciferase as the level of induction.
Values represent the mean of triplicate samples and are representatives of 3 independent experiments. (B) PC12 cells were transiently transfected with a control vector (a, b), or with a vector encoding a kinase-deficient MAPK mutant (dnMAPK; c, d) along with 1/10 of the molar amount of a GFP vector as indicated. One day post transfection, cells were incubated with normal growth medium containing NGF (100 ng/ml; a, c), or NGF plus CGS21680 (CGS, 1 µM; b, d), as indicated for 72 h. (C) PC12 cells were transiently transfected with a control vector (e, f), or with a vector encoding dnMAPK (g, h) along with 1/10 of the molar amount of a GFP vector as indicated. One day post transfection, cells were pretreated with an A2A-R-specific antagonist (CSC, 10 µM) for 30 min before further incubation with normal growth medium containing NGF (e, g) or NGF plus CGS (f, h) as indicated for 3 days. Data shown are representative images of the transfected cells identified by GFP expression. Scale bars represent 50 µm. (D) NGF-evoked neurite-bearing cells transfected with a control vector (open bars) or with a vector encoding dnMAPK (shaded bars) in the presence of the indicated reagent(s) were quantified. Totals of at least 100 GFP-positive cells were scored in each condition. Data represent the mean ± SEM values from at least 3 independent experiments. *p < 0.001 compared to the corresponding non-CGS treated sample (two-way analysis of variance). †Specific comparison between cells treated with or without CSC (p < 0.001; two-way analysis of variance). ‡Specific comparison between cells transfected with dnMAPK or a control vector (p < 0.001; two-way analysis of variance). §Specific comparison between cells transfected with indicated plasmid or a control vector (p < 0.001; two-way analysis of variance).

Fig. 4. PKA mediates A2A-R's action in rescuing NGF-induced neurite outgrowth abated by dnMAPK. (A) PC12 cells were transiently transfected with an empty vector (open bars) or dnMAPK (shaded bars) along with 1/10 of the molar amount of a GFP vector. One day post transfection, cells were pretreated with or without H89 (10 µM) for
30 min before the addition of NGF (100 ng/ml), NGF plus CGS21680 (CGS, 1 µM), or NGF plus forskolin (FK, 10 µM) as indicated for 72 h. (B) PC12 cells were transiently transfected with a GFP vector, a construct encoding dnMAPK (shaded bars) or a control vector (open bars), and a construct encoding PKI or a control vector in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated in normal growth medium containing no additive, NGF, or NGF plus CGS as indicated for 3 days. (C) A123 cells were transiently transfected with a control vector (open bars) or with a vector encoding dnMAPK (shaded bars) along with 1/10 of the molar amount of a GFP vector as indicated. One day post transfection, cells were incubated with normal growth medium containing NGF (100 ng/ml) or NGF plus CGS (1 µM) as indicated for 72 h. NGF-evoked neurite-bearing cells transfected with a control vector (open bars) or with a vector encoding dnMAPK (shaded bars) in the presence of the indicated reagent(s) or the indicated plasmid were quantified. Totals of at least 100 GFP-positive cells were scored in each condition. Data represent the mean ± SEM values from at least 3 independent experiments. Values represent the mean ± SEM. Specific comparison between cells treated with or without CGS (p < 0.001; two-way analysis of variance). Specific comparison between cells treated with or without H89 (p < 0.001; two-way analysis of variance). Specific comparison between cells transfected with dnMAPK or a control vector (p < 0.001; two-way analysis of variance). Specific comparison between cells transfected with indicated plasmid or a control vector (p < 0.001; two-way analysis of variance). Specific comparison between cells treated with or without forskolin (p < 0.001; two-way analysis of variance).

Fig. 5. A PKA-selective inhibitor blocks the phosphorylation of CREB induced by A2A-R stimulation. (A) PC12 cells were treated with or without H89 (10 µM) for 30 min before the addition of CGS (1 µM) for 10 min. Phosphorylated CREB (P-CREB, upper panel) and total CREB protein (lower panel) in cell lysates were quantified by Western
blot analysis. Phosphorylation of CREB at 133Ser was quantified by quantitative computed densitometry of the immunoreactive bands of p-CREB and total CREB recorded on Kodak XAR-5 film from 3 independent experiments using the image analysis software package, ImageQuant v.3.15 (Molecular Dynamics). (B) Values for CREB phosphorylation extent [(integrated absorbance units of P-CREB signal ÷ CREB signal of the indicated sample) x 100] are expressed as percentages of the phosphorylation of CREB by CGS alone. *Specific comparison between cells treated with or without H89 (p < 0.001; one-way analysis of variance).

Fig. 6. Stimulation of A2A-R leads to activation of CREB in the presence of a MEK inhibitor. (A) PC12 cells were pretreated with or without PD98059 (PD; 100 µM) for 30 min before the addition of CGS (1 µM) and/or NGF (100 ng/ml) as indicated for 10 min. Phosphorylated CREB (P-CREB, upper panel) and total CREB protein (lower panel) in cell lysates were quantified by Western blot analysis. Data shown are representative of results from 3 to 5 independent experiments. (B) Values for phosphorylation extent of CREB (i.e., integrated absorbance units of phosphorylation signal ÷ the total protein signal) are expressed as percentages of the phosphorylation of CREB by NGF alone. The data were generated by quantitative computing densitometry of autoradiograms from 3 to 5 independent experiments using the image analysis software package, ImageQuant v.3.15 (Molecular Dynamics). Data points are the mean ± SEM values. *Specific comparison between samples treated with or without PD98059 (p < 0.001; two-way analysis of variance). *Specific comparison to control samples (no addition; p < 0.001; two-way analysis of variance). bSpecific comparison between samples treated with or without CGS in the presence of NGF (p < 0.005; two-way analysis of variance). (C) Expression vectors encoding Gal4-DNA binding domain (dbd) or chimeric Gal4dbd-CREB protein were cotransfected with a SV40 promoter-driven R-luciferase (as an internal control) into PC12 cells together with an F-luciferase reporter gene driven by 5 copies of the Gal4-binding element together. Twenty-four hours post transfection, cells were treated with the indicated reagent for 3 h. The activity of F-luciferase was normalized to the activity of R-luciferase, and subtracted from those in the absence of stimuli. The normalized basal F-luciferase activities in PC12 cells in the absence or
presence of PD were $0.0114 \pm 0.0016$ and $0.0322 \pm 0.0029$, respectively. Values represent the mean of triplicate samples and are representative of 3 independent experiments.

Fig. 7. **Expression of a dominant negative CREB mutant (CREBm1 and CREBR287L) suppresses $A_2A$-R's action in rescuing NGF-induced neurite outgrowth abated by dnMAPK** (A) Expression vectors encoding CREBm1 or CREBR287L proteins were cotransfected with an F-luciferase reporter gene driven by 3 copies of CRE, and TK promoter-driven R-luciferase (as an internal control) into PC12 cells. Forty-eight hours post transfection, cells were treated with or without CGS (1 $\mu$M) as indicated for 3 h. The activity of F-luciferase was normalized to the activity of R-luciferase as the level of induction. Values represent the mean of triplicate samples and are representative of 3 independent experiments. (B) PC12 cells were transiently transfected with a GFP vector, a control vector (open bars) or a construct encoding dnMAPK (shaded bars), and a construct encoding a negative CREB mutant (CREBm1) or an empty vector in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing NGF (100 ng/ml) or NGF plus CGS21680 (CGS, 1 $\mu$M) as indicated for 72 h. (C) PC12 cells were transiently transfected with a GFP vector, a construct encoding a negative CREB mutant (CREBR287L), and an empty vector or a construct encoding dnMAPK (shaded bar) or a control vector (open bar) in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing NGF (100 ng/ml), or NGF plus CGS21680 (CGS, 1 $\mu$M) as indicated for 72 h. Totals of at least 100 GFP-positive cells were scored for each condition. Data represent the mean $\pm$ SEM values from at least 3 independent experiments. $^b$Specific comparison between cells treated with or without CGS ($p < 0.001$; two-way analysis of variance). $^d$Specific comparison between cells transfected with dnMAPK or a control vector ($p < 0.001$; two-way analysis of variance). $^e$Specific comparison between cells transfected with indicated plasmid or an empty vector ($p < 0.001$; two-way analysis of variance).
Fig. 8. Expression of a constitutively active CREB mutant (CREB-VP16) rescues NGF-induced neurite outgrowth abated by a dominant negative MAPK mutant. (A) PC12 cells were transiently transfected with a GFP vector, a control vector, and a construct encoding a constitutively active CREB mutant (CREB-VP16; c, d) or an empty vector (VP16; a, b) in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing no additive (a, c) or NGF (100 ng/ml; b, d) as indicated for 72 h. (B) PC12 cells were transiently transfected with a GFP vector, a construct encoding dnMAPK, and a construct encoding a constitutively active CREB mutant (CREB-VP16; g, h) or an empty vector (VP16; e, f) in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing no additive (e, g) or NGF (100 ng/ml; f, h) as indicated for 72 h. Data shown are representative images of transfected cells identified by GFP expression. Scale bars represent 50 µm. (C) Neurite-bearing cells transfected with the indicated plasmid and a control vector (open bars) or a vector encoding dnMAPK (shaded bars) in the presence of NGF were quantified. Totals of at least 100 GFP-positive cells were scored for each condition. Data represent the mean ± SEM values from at least 3 independent experiments. *Specific comparison between cells transfected with dnMAPK or a control vector (p < 0.001; two-way analysis of variance). **Specific comparison between cells transfected with the CREB-VP16 plasmid or the control VP16 vector (p < 0.001; two-way analysis of variance).

Fig. 9. Expression of a dominant negative PI3K mutant (Δp85) suppresses A2A-R's action in rescuing NGF-induced neurite outgrowth abated by dnMAPK. (A) PC12 cells were transfected with an HA-Akt construct, and an empty vector or a vector encoding Δp85 in the molar amounts of 7 and 7. Forty-eight hours post transfection, cells were treated with or without NGF (100 ng/ml) for 15 min as indicated. Total lysates collected from the indicated cells were immunoprecipitated with an anti-HA antibody followed by Western blot analysis to determine the phosphorylation of HA-Akt at Ser^{473} (upper panel) and total HA-Akt protein (lower panel). Data shown are representative of 3 independent experiments. (B) PC12 cells were transiently transfected with a GFP vector, a control vector, and a construct encoding a negative PI3K mutant (Δp85; d-f) or an
empty vector (a-c) in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing no additive (a, d), NGF (100 ng/ml; b, e), or NGF plus CGS21680 (CGS, 1 μM; c, f) as indicated for 72 h. (C) PC12 cells were transiently transfected with a GFP vector, a construct encoding dnMAPK, and a construct encoding Δp85 (j-l) or an empty vector (g-i) in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing no additive (g, j), NGF (100 ng/ml; h, k), or NGF plus CGS (1 μM; i, l) as indicated for 72 h. Data shown are representative images of transfected cells identified by GFP expression. Scale bars represent 50 μm. (D) NGF-evoked neurite-bearing cells transfected with the indicated plasmid and a control vector (open bars) or a vector encoding dnMAPK (shaded bars) in the absence or presence of CGS as indicated were quantified. Totals of at least 100 GFP-positive cells were scored for each condition. Data represent the mean ± SEM values from at least 3 independent experiments. aSpecific comparison between cells treated with or without CGS (p < 0.001; two-way analysis of variance). bSpecific comparison between cells transfected with dnMAPK or a control vector (p < 0.001; two-way analysis of variance). cSpecific comparison between cells transfected with indicated plasmid encoding Δp85 or an empty vector (p < 0.001; two-way analysis of variance).

Fig. 10. A constitutively positive mutant of PI3K (P110*) synergized with cAMP-mediated signals to induce neurite outgrowth. (A) PC12 cells were transiently transfected with the control vector or a construct encoding a constitutively active mutant of PI3K (p110*). Twenty-four to 48 h post transfection, cells were harvested and analyzed for the expression of p110* with an anti-myc monoclonal antibody (upper panel). Even loading of the gel was demonstrated by the expression of actin visualized using an anti-actin antibody (lower panel). (B) PC12 cells were transfected with an HA-Akt construct, and an empty vector or a vector encoding p110* in the molar amounts of 7 and 7. Forty-eight hours post transfection, total lysates collected from the indicated cells were immunoprecipitated with an anti-HA antibody followed by Western blot analysis to determine the phosphorylation of HA-Akt at Ser473 (upper panel) and total HA-Akt protein (lower panel). Data shown are representative of 3 independent experiments. (C)
PC12 cells were transiently transfected with the control vector or a construct encoding a constitutively active mutant of PI3K (p110*) along with 1/10 of the molar amount of a GFP vector. One day post transfection, cells were treated with normal growth medium containing no additive (a, d), CGS21680 (CGS, 1 μM; b, e), or forskolin (FK, 10 μM; c, f), as indicated for 72 h. Data shown are representative images of transfected cells identified by GFP expression. Scale bars represent 50 μm. (D) Neurite-bearing cells transfected with the control vector (closed bars) or with a vector encoding p110* (open bars) in the presence of the indicated reagent(s). Data are expressed as the percentage of neurite-bearing cells, and represent the mean ± SEM values from 3 different fields. Totals of at least 300 GFP-positive cells were scored for each condition. Data points are the mean ± SEM values from 3 independent experiments. (E) PC12 cells were transiently transfected with a GFP vector, a p110* construct, and a dnMAPK encoding construct or a CREBR287L-encoding construct or an empty vector, in the molar amounts of 1, 7, and 7, respectively. (F) PC12 cells were transiently transfected with a GFP vector, a p110* construct or an empty vector, a CREB-VP16 construct or an empty vector, as indicated in the molar amounts of 1, 7, and 7, respectively. One day post transfection, cells were incubated with normal growth medium containing no additive, CGS21680 (CGS, 1 μM), or forskolin (FK, 10 μM) as indicated for 72 h. Data are expressed as the percentage of neurite-bearing cells, and represent the mean ± SEM values from 3 different fields. Totals of at least 300 GFP-positive cells were scored for each condition. Data points are the mean ± SEM values from 3 independent experiments. Specific comparison between cells treated with or without CGS (p < 0.001). Specific comparison between cells transfected with a indicated plasmid or an empty vector (p < 0.001; two-way analysis of variance). Specific comparison between cells treated with or without forskolin (p < 0.001).

Fig. 11. **TrkA is not required for the neurite outgrowth process induced by simultaneous activation of the PI3K- and the A2A-R/cAMP-mediated pathways.**

PC12 cells were transiently transfected with a p110* construct (shaded bar) or an empty vector (open bar) along with 1/10 of the molar amount of a GFP vector. One day post transfection, cells were treated with or without K252a (100 nM) for 30 min before the
addition of normal growth medium containing no additive or CGS21680 (CGS, 1 µM) as indicated for 72 h. Data are expressed as the percentage of neurite-bearing cells, and represent the mean ± SEM values from 3 different fields. Totals of at least 300 GFP-positive cells were scored for each condition. Specific comparison between cells treated with or without CGS (p < 0.001). Specific comparison between cells treated with K252a or not (p < 0.001; two-way analysis of variance).

Fig. 12. Schematic representation of signaling pathways that mediate A2A-R’s action in rescuing NGF-induced neurite outgrowth abated by blockage of the MAPK cascade.
Table 1. PKC is not involved in the rescue effect of A2A-R in PC12 cells. PC12 cells at 20% confluence were treated with or without the indicated reagents (NGF, 100 ng/ml; PD98059, 20 μM; CGS, 1 μM; CHE, 1 μM) for 3 days. Neurite-bearing cells were normalized to the number of total cells and are expressed as a percentage of the NGF-treated group in the absence of other reagents. The percentage of neurite-bearing cells under the indicated condition is normalized with that of the control group (NGF-treated cells transfected with an empty vector; 27.1% ± 1.5%) and is expressed as a percent (%) of NGF-induced neurite bearing cells. Data points are the mean ± SEM values from 3 independent experiments. "Specific comparison between cells treated with or without PD98059 (p < 0.05; two-way analysis of variance). "Specific comparison between cells treated with or without CGS (p < 0.05; two-way analysis of variance). "Specific comparison between cells treated with or without CHE (p < 0.05; two-way analysis of variance).

<table>
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<tr>
<th>Inhibitor</th>
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<th>PD</th>
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<tr>
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<td>100</td>
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<td></td>
<td>+</td>
<td>135±14</td>
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<tr>
<td>CHE</td>
<td>-</td>
<td>75 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>+</td>
<td>131±47</td>
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Fig. 1

A

B
Fig. 2

| NGF | - | + | - | + | - | + | - | + |
| CGS | - | - | + | + | - | - | + | + |
| PD  | - | - | - | - | + | + | + | + |

P-MAPK

MAPK
Fig. 3

A

![Graph showing relative luciferase activity](image)

B

![Images of cell cultures](image)

C

![Images of cell cultures](image)

D

![Graph showing percentage of neurite-bearing cells](image)
**Fig. 4**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)
Fig. 5

A

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<tr>
<th></th>
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<tr>
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B

![Graph showing % CREB phosphorylation with CGS and H89 treatments]
Fig. 6

A

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<tr>
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P-CREB

CREB

B

CON

PD98059

% CREB phosphorylation

\[ \text{CON} \quad \text{NGF} \quad \text{CGS} \quad \text{NGF/CGS} \]

C

\[ \text{dbdCREB/CON} \quad \text{dbdCREB/PD} \]

Relative luciferase activity

\[ \text{NGF} \quad \text{CGS} \quad \text{NGF/CGS} \]
Fig. 7

A

![Bar graph showing relative luciferase activity](image)

B

![Bar graph showing % Neurite-Bearing Cells](image)

C

![Bar graph showing % Neurite-Bearing Cells](image)
Fig. 8

A  

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>VP16</td>
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<tr>
<td>CREB-VP16</td>
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B  

<table>
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<tr>
<td>CREB-VP16</td>
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C  

Bar chart showing the percentage of neurite-bearing cells. The chart compares Vector and dnMAPK groups under VP16 and CREB-VP16 conditions.
Fig. 9

A

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<tbody>
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<td>NGF</td>
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<tr>
<td>P-Akt</td>
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<tr>
<td>Akt</td>
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</tr>
</tbody>
</table>

B

pcDNA3

Δp85

C

pcDNA3

Δp85

D

% Neurite-Bearing Cells

Vector

dnMAPK

Plasmid | Vector | Δp85 | Vector | Δp85 |
--------|--------|------|--------|------|
CGS     | -      | -    | +      | +    |
        | d      | c    | d      | d    |

Vector

Δp85
**Fig. 10**

Panel D:

- Neurite-Bearing Cells
- Vector
- p110

Panel E:

- Neurite-Bearing Cells
- Vector
- CREB287L
- dnMAPK

Panel F:

- Neurite-Bearing Cells
- Vector
- p110
- CREB-VP16
Fig. 11

% Neurite-Bearing Cells

<table>
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<tr>
<th>Vector</th>
<th>p110*</th>
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</thead>
<tbody>
<tr>
<td>b,l</td>
<td>b,l</td>
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</tbody>
</table>

K252a - CON - CGS

- - + - +

0 2 4 6 8 10
Essential role of CREB activation by A2A adenosine receptors in rescuing the NGF-induced neurite outgrowth impaired by blockage of the MAPK cascade
Hsiao-Chun Cheng, Hsiu-Ming Shih and Yijuang Chern

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