

Original Article

# Adenosine A<sub>1</sub> receptor-mediated transactivation of the EGF receptor produces a neuroprotective effect on cortical neurons *in vitro*

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**Aim:** To understand the mechanism of the transactivation of the epidermal growth factor receptor (EGFR) mediated by the adenosine A<sub>1</sub> receptor (A<sub>1</sub>R).

**Methods:** Primary cultured rat cortical neurons subjected to oxygen-glucose deprivation (OGD) and HEK293/A<sub>1</sub>R cells were treated with the A<sub>1</sub>R-specific agonist N<sup>6</sup>-cyclopentyladenosine (CPA). Phospho-EGFR, Akt, and ERK1/2 were observed by Western blot. An interaction between EGFR and A<sub>1</sub>R was detected using immunoprecipitation and immunocytochemistry.

**Results:** The A<sub>1</sub>R agonist CPA causes protein kinase B (Akt) activation and protects primary cortical neurons from oxygen-glucose deprivation (OGD) insult. A<sub>1</sub>R and EGFR co-localize in the membranes of neurons and form an immunocomplex. A<sub>1</sub>R stimulation induces significant EGFR phosphorylation via a PI3K and Src kinase signaling pathway; this stimulation provides a neuroprotective effect in cortical neurons. CPA leads to sustained phosphorylation of extracellularly regulated kinases 1 and 2 (ERK1/2) in cortical neurons, but only to transient phosphorylation in HEK 293/A<sub>1</sub>R cells. The response to the A<sub>1</sub>R agonist is mediated primarily through EGFR transactivation that is dependent on pertussis toxin (PTX)-sensitive G<sub>i</sub> protein and metalloproteases in HEK 293/A<sub>1</sub>R.

**Conclusion:** A<sub>1</sub>R-mediated EGFR transactivation confers a neuroprotective effect in primary cortical neurons. PI3 kinase and Src kinase play pivotal roles in this response.

**Keywords:** adenosine A<sub>1</sub> receptor; epidermal growth factor receptor; transactivation; Akt; Src kinase; ERK1/2; G<sub>i</sub> protein; metalloprotease; PI3K

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## Introduction

Extracellular brain adenosine concentrations increase 30- to 100-fold after a pathological trauma such as ischemia, epileptic seizure, or head injury. The accumulation of adenosine during these pathologies protects the brain from injury<sup>[1]</sup>. In the extracellular space, adenosine modifies cell functioning by acting on adenosine receptors (A<sub>1</sub>R, A<sub>2a</sub>R, A<sub>2b</sub>R, A<sub>3</sub>R)<sup>[2, 3]</sup>. The activation of adenosine A<sub>1</sub> receptor (A<sub>1</sub>R) is known to mediate neuronal and myocardial protection<sup>[1]</sup>.

Serine/threonine protein kinase B (PKB, also called Akt) is part of an endogenous neuroprotection pathway in both necrotic and apoptotic models of neuronal cell death<sup>[4]</sup>, and an A<sub>1</sub>R agonist has been reported to induce Akt phosphorylation

in DDT<sub>1</sub>MF-2<sup>[5]</sup> and the rat hippocampus<sup>[6]</sup>. However, the signaling mediators of the A<sub>1</sub>R-induced Akt activation pathway have not yet been identified in the neurons.

Recently, ligand-independent transactivation of receptor tyrosine kinases (RTKs) was shown to be an important factor in the regulation of GPCR-mediated cellular differentiation, growth, and proliferation, in particular via ERK1/2 activation<sup>[7, 8]</sup>. Previous studies have shown that, depending on the cell type, epidermal growth factor receptor (EGFR) transactivation by GPCR agonists may be inhibited by PTX, the sequestration of free G protein βγ subunits, Ca<sup>2+</sup> chelators, or the inhibition of PKC, metalloproteinase, or Src<sup>[7]</sup>.

In addition to RTK transactivation in the absence of growth factors, some GPCR agonists can also promote the synergistic regulation of RTKs upon concomitant stimulation with an RTK ligand. In human airway smooth muscle cells, various GPCR agonists, which are often weak mitogens themselves, have been shown to promote the synergistic stimulation of cell proliferation when combined with growth factors such as EGF

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or platelet-derived growth factor (PDGF)<sup>[9]</sup>. Previous reports have shown that the GPCR agonist thrombin can potentiate the mitogenic effects of EGF in human airway smooth muscle<sup>[10]</sup>. EGFR has been recognized as a convergence point for diverse signal transduction pathways<sup>[11]</sup>. Therefore the ability to regulate EGFR activity is critical for cell survival, proliferation and differentiation.

Some of the important players in the A<sub>1</sub>R signaling pathway are known, but the order in which they are activated is still unclear. For example, it is not known how RTK transactivation is involved and when in the cascade this event occurs relative to the activation of PI3K and other mediators. We examined EGFR transactivation induced by A<sub>1</sub>R stimulation in primary cultured cortical neurons and HEK293 cells stably transfected with A<sub>1</sub>R, focusing on Akt and ERK1/2 activation. This is the first study to elucidate how EGFR transactivation is involved in A<sub>1</sub>R-mediated neuroprotection in primary cortical neurons.

## Materials and methods

### Materials

Neurobasal B27 and G418 were purchased from Gibco Invitrogen (Grand Island, NY). Pertussis toxin (PTX), EGF, N<sup>6</sup>-cyclopentyladenosine (CPA) and Hoechst 33258 were from Sigma (St Louis, MO). 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), (2R)-[(4-biphenyl)sulfonyl]amino]-N-hydroxy-3-phenylpropionamide (BiPS), and wortmannin (Wort) were obtained from Calbiochem (San Diego, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Bio Basic Inc (Markham Ontario, Canada). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). Gel/Mount mounting media was obtained from Biomed (Foster City, CA). ERK1/2, Akt, phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473) and phospho-EGFR (Tyr1068) antibodies were from Cell Signaling Technology, Inc (Beverly, MA). Anti-phosphotyrosine (p-Tyr, 4G10) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). EGFR antibodies were obtained from Cell Signaling Technology and Upstate Biotechnology (Lake Placid, NY). Anti-A<sub>1</sub>R antibody was from Affinity BioReagents (Golden, CO).

### Primary rat cortical neuronal cultures

Dissociated primary cultures of cortical neurons from embryonic day 18 (E18) rats were prepared from timed-pregnant Sprague-Dawley rats as described previously with minor modifications<sup>[12]</sup>. Fetuses were removed under sterile conditions and kept in iced D-Hanks' solution for the microscopic dissection of the cortex. The meninges were removed, and the tissue was minced briefly with fine forceps and then triturated with a fire-polished Pasteur pipette. Cells were counted and plated on six-well culture plates or coverslips coated with poly-D-lysine in DMEM with 10% fetal bovine serum. The medium was replaced with serum-free B27/neurobasal medium supplemented with 0.5 mmol/L glutamine

and antibiotics 5 h later. Cultures were maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Experiments were conducted 9–10 d after plating. For experiments in which enzyme inhibitors or receptor antagonists were tested, neurons were pretreated with various agents for 30 min before the addition of CPA, except in the case of the ADP-ribosylating factor of the inhibitory guanosine nucleotide binding protein (G<sub>i</sub>), PTX, which was added overnight (16 h). These treatments included selective inhibitors of Src kinase: PP2, PI3K, wortmannin (Wort), metalloprotease, BiPS, and a selective antagonist of A<sub>1</sub>R, DPCPX.

### HEK293/A<sub>1</sub>R cell cultures

A human embryonic kidney 293 cell line that stably expresses human adenosine A<sub>1</sub> receptor (HEK293/A<sub>1</sub>R cell) was established previously and maintained in our lab<sup>[13]</sup>. Cells were cultured at 37 °C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 200 µg/mL G418.

### Oxygen glucose deprivation (OGD) protocol and cell viability assay

OGD was carried out on 9 DIV (days *in vitro*) cultures. Briefly, neurons were pretreated with the indicated compounds in B27/neurobasal medium for 4 h. Then the medium (conditioned medium) was collected. Cells were washed once and maintained in glucose-free buffered Hanks' solution (in mmol/L: 5.26 KCl, 0.43 KH<sub>2</sub>PO<sub>4</sub>, 132.4 NaCl, 4.09 NaHCO<sub>3</sub>, 0.33 Na<sub>2</sub>HPO<sub>4</sub>, 2 CaCl<sub>2</sub>, and 20 mmol/L HEPES, pH 7.4). This medium had been bubbled with 95% N<sub>2</sub>/5% CO<sub>2</sub> at 3 liter/min for 5 min and pre-warmed to 37°C. Hypoxia was induced by placing cells at 37 °C in a humidified, sealed chamber that had been flushed with 95% N<sub>2</sub>/5% CO<sub>2</sub> for 5 min. The indicated drugs were also included in the OGD solution during the OGD period. At the end of the OGD period (4 h), cultures were returned to the conditioned medium for another 12 h. Cell viability was assessed by metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described previously<sup>[14]</sup>. 12 h after OGD treatment, the MTT solution (0.5 mg/mL) was added to each well, and cells were maintained in growth medium for 4 h. After solubilization with DMSO, the absorbance of the formazan dye was measured using a microplate reader (NovoStar, BMG) at 595 nm. In each experiment, cultures exposed to OGD were always compared with normoxic controls, which were supplied with buffered Hanks' solution containing 5.5 mmol/L glucose and maintained in standard incubation conditions. For the assays, four wells were used for each condition, and each experiment was repeated at least five times. The viability of normoxia cultures that were assessed with this method was unaffected during the duration of the experiment. The results are expressed relative to the controls specified in each experiment and were subjected to statistical analysis.

### Coimmunoprecipitation and immunoblotting

Cells were serum/B27-starved for 18 h at 37 °C before treat-

ment. Cell lysates from cultured cortical neurons were prepared by incubating in modified radioimmunoprecipitation assay (RIPA) lysis buffer (Tris-HCl 50 mmol/L, pH 7.4, NaCl 150 mmol/L, EDTA 1 mmol/L, Na-deoxycholate 0.25%, NP-40 1%, PMSF 1 mmol/L, Na<sub>3</sub>VO<sub>4</sub> 1 mmol/L, NaF 1 mmol/L, aprotinin 10 µg/mL, leupeptin 5 µg/mL, and pepstatin 5 µg/mL). Clarified lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to PVDF membranes as described previously<sup>[15]</sup>. Blots were incubated overnight at 4 °C with primary antibodies. After probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the immunoreactive proteins were visualized with enhanced chemiluminescence kit (Pierce). In some cases, blots were stripped and reprobed with other antibodies. The interaction between A<sub>1</sub>R and EGFR was examined by coimmunoprecipitation. Co-IP was performed as previously reported. Briefly, neuronal cultures were prepared by incubated in lysis buffer (300 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, 10 mmol/L EDTA, 50 mmol/L Tris, pH 7.4) containing 100 mmol/L iodoacetamide, 10 µg/mL aprotinin, and 5 µg/mL leupeptin for 45 min on ice. Clarified lysates were immunoprecipitated by incubated overnight at 4 °C with an anti-A<sub>1</sub>R antibody (Affinity BioReagents, Golden, Co) or anti-EGFR antibody and then incubated with A-Sepharose beads. Equivalent amounts of protein were analyzed for each condition. Normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control. The beads were washed three times with lysis buffer, and the immune complexes were boiled in SDS-sample buffer and loaded on SDS-PAGE gels for immunoblot analysis. The immunoreactive protein bands were detected by enhanced chemiluminescence (Pierce).

#### Immunocytochemistry

Immunochemical processing was performed as described previously with minor modifications<sup>[15]</sup>. Briefly, cultured cortical neurons were fixed in 4% paraformaldehyde for 30 min at 4 °C. After two rinses in PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature. Cells were blocked for 1 h in blocking buffer (5% normal goat serum, 1% BSA, 0.1% Triton X-100, and 0.1% sodium azide in PBS) before incubation with anti-A<sub>1</sub>R and anti-EGFR antibodies diluted in blocking buffer at 4 °C overnight. Cells were washed three times with PBS and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG (1:100) (Molecular probes, Eugene, OR) for 1.5 h at room temperature. The images were acquired with a confocal microscope (Leica, Germany).

#### Statistics

The data are presented as means±SEM and were compared with a one-way ANOVA followed by a Bonferroni *post hoc* test using the GraphPad InStat statistical program. The level of the statistical significance was set at  $P < 0.01$  and  $P < 0.05$ . The data presented are from at least three separate experiments.

## Results

### The A<sub>1</sub>R agonist CPA promotes survival in cultured cortical neurons during OGD

Many previous investigations, both *in vivo* and *in vitro*, have documented the neuroprotective effects of A<sub>1</sub>R<sup>[16]</sup>. The inhibition of glutamate release, hyperpolarization of neurons, and the direct inhibition of certain kinds of Ca<sup>2+</sup> channels have been shown to mediate the neuroprotective effect. We sought to determine whether A<sub>1</sub>R agonists protect cortical neurons in cerebral ischemia and, if so, to ascertain the associated upstream signaling mechanism. We used an *in vitro* model of cerebral ischemia, in which rat primary cultured cortical neurons are exposed to a combination of oxygen and glucose deprivation (OGD), which resembles the *in vivo* conditions. OGD exposure for 4 h causes a significant drop in neuronal cell viability, as shown by phase-contrast morphology and MTT reduction compared to normoxia control. This cell loss was nearly completely reversed by a pretreatment with an A<sub>1</sub>R agonist, N<sup>6</sup>-cyclopentyladenosine (CPA) (1 µmol/L, 4 h) (Figure 1). If treated with DPCPX, an antagonist of A<sub>1</sub>R, the protective effect provided by CPA is abolished. These results clearly indicate that CPA can promote the survival of cortical neurons during OGD.

### EGFR signaling is necessary for A<sub>1</sub>R-mediated neuroprotection

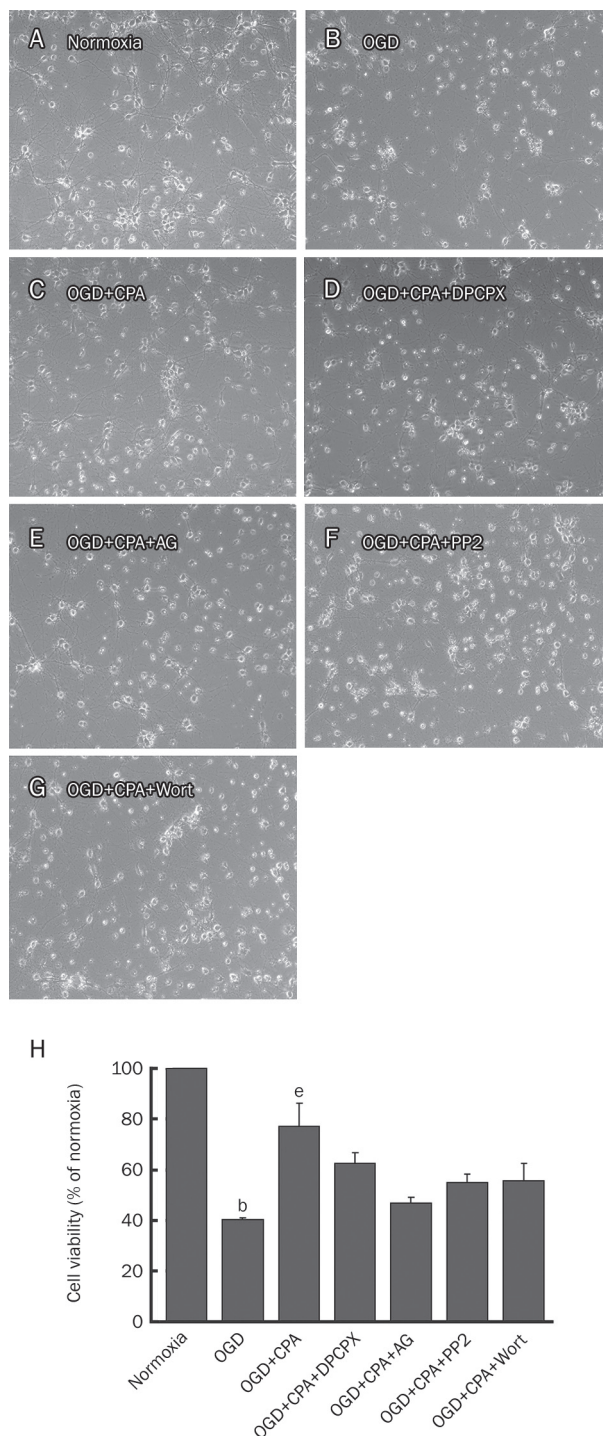
EGFR has proved to be an important factor in the regulation of GPCR-mediated cellular differentiation, growth and proliferation<sup>[7, 11, 17, 18]</sup>. To determine the involvement of EGFR signaling in CPA-induced neuronal survival, we added the selective EGFR kinase inhibitor, AG1478 (1 µmol/L), to block EGFR kinase activity. Strikingly, AG1478 was found to abolish the neuroprotection provided by CPA. Similar effects were observed upon inhibition of phosphoinositide 3-kinase (PI3K) by wortmannin (Wort) or Src kinase by PP2 (Figure 1). The cell viability assay after OGD insult indicates that CPA-induced neuroprotection may be mediated by EGFR, Src kinase and PI3K (Figure 1H).

### Characterization of cortical neurons in culture

We next sought to investigate how EGFR signaling is involved in A<sub>1</sub>R agonist-mediated neuroprotection. We used immunocytochemistry staining with antibodies specific for EGFR and A<sub>1</sub>R to address the question of whether the receptors for EGF and A<sub>1</sub>R are expressed in the same cortical neurons. Cultures were grown in Neurobasal/B27 serum-free medium for 9 to 11 days and yielded virtually pure neurons. We found that most of the cells were stained by both EGFR and A<sub>1</sub>R antibodies (Figure 2A). This result shows that the two receptors co-localize in the membrane of cortical neurons (Figure 2C), raising the possibility that neurons are capable of responding to both A<sub>1</sub>R agonists and EGF.

### A<sub>1</sub>R forms a complex with EGFR in cultured cortical neurons, and A<sub>1</sub>R stimulation induces EGFR transactivation

To clarify the role of EGFR in mediating the A<sub>1</sub>R-induced



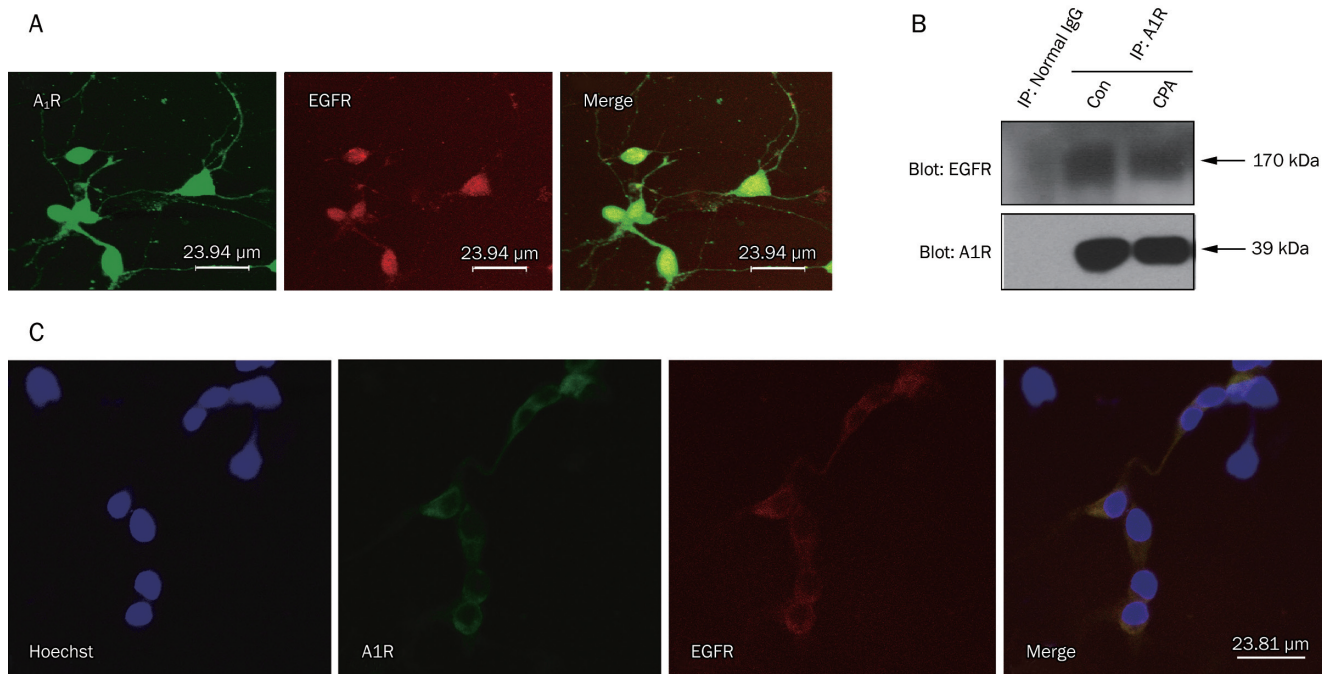
**Figure 1.** Neuroprotective effect of adenosine  $A_1$  receptor agonist CPA in rat cortical neurons exposed to OGD. Phase-contrast micrographs (A–G) of representative cultures are shown. Cortical neurons were pretreated with the indicated compounds (C–G) or the vehicle (A and B) dissolved in neurobasal medium for 12 h and then exposed to OGD for 4 h. At the end of OGD exposure, cells were then maintained in conditional medium for another 16 h. Cell viability was assessed with the MTT assay (H) and calculated as the percentage of the normoxia control. Means $\pm$ SEM from at least three independent experiments. <sup>b</sup> $P$ <0.05 compared to the normoxia group; <sup>e</sup> $P$ <0.05 compared with the OGD treatment group (one-way ANOVA followed by Bonferroni *post hoc* test).

neuroprotective effects, we looked for a physical association between these two receptors. After exposing cortical neurons to CPA or the vehicle, we prepared cell extracts and used a specific anti- $A_1$ R antibody or normal IgG to perform immunoprecipitations and immunoblotting for EGFR. We found that EGFR co-immunoprecipitated with  $A_1$ R and that the extent of association between these two receptors did not change upon CPA treatment (1  $\mu$ mol/L, 15 min) (Figure 2B). EGFR did, however, show an increase in tyrosine phosphorylation as indicated by the anti-phospho-Tyr antibody (Figure 6A). These results indicate that  $A_1$ R and EGFR form a complex and that  $A_1$ R stimulation may induce EGFR phosphorylation (EGFR transactivation). As shown in Figure 6A, the specific EGFR kinase inhibitor, AG1478, weakens EGFR transactivation following  $A_1$ R stimulation, suggesting that EGFR transactivation is mediated by an intrinsic EGFR tyrosine kinase.

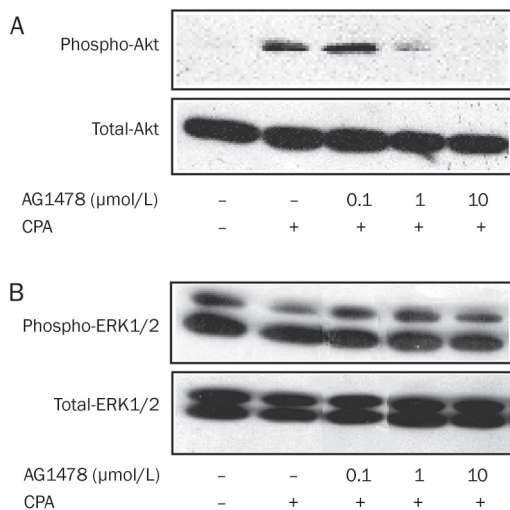
#### EGFR transactivation mediated by $A_1$ R stimulation is dependent on PI3K and Src kinase in cortical neurons

The results discussed above suggest that the neuroprotective activity of CPA in cortical neurons depends significantly on EGFR and PI3K. We wanted to know whether the events that are downstream of EGFR, ERK1/2, and the PI3K target, Akt, which has been reported to be important in mediating survival in many cell types, function in EGFR transactivation. Akt is activated by the phosphorylation of Thr308 in the catalytic loop and Ser473 in the C-terminal domain; ERK1/2 is activated by phosphorylation at Thr202/Tyr204<sup>[19]</sup>. We wanted to investigate whether the neuroprotectant CPA induced the phosphorylation of Akt and ERK1/2 in cortical neurons. Antibodies that specifically recognize the phosphorylated form of Akt or ERK1/2 were used to monitor Akt and ERK1/2 activation. We found that Akt was indeed significantly phosphorylated at Ser473 in cortical neurons upon exposure to 1  $\mu$ mol/L CPA for 5 min. A pretreatment with AG1478 reversed Akt activation, as shown in Figure 3A. An analysis of the dose-response (ranging from 0.1  $\mu$ mol/L to 10  $\mu$ mol/L) indicates that 1  $\mu$ mol/L of AG1478 is sufficient to abolish the Akt phosphorylation induced by CPA. This result suggests that EGFR is involved in the induction of Akt activation by CPA stimulation. CPA also activates ERK1/2 (Figure 3B). Unlike Akt, however, ERK1/2 activation is not affected by AG1478 (Figure 3B).

We next attempted to determine the signal mediators that connect  $A_1$ R to Akt activation. Consistent with the above finding that EGFR is necessary for  $A_1$ R-induced neuroprotection, the involvement of Akt activation in EGFR transactivation by  $A_1$ R stimulation was supported by finding a concentration-dependent inhibition of these responses after pretreatment with EGFR kinase inhibitor AG1478. The activation of RTKs, such as EGFR and PDGFR, often triggers the recruitment of PI3K and the activation of Akt<sup>[20]</sup>. We pretreated cortical neurons with the selective PI3K inhibitor wortmannin (Wort, 100 nmol/L, 30 min) and then stimulated with CPA (1  $\mu$ mol/L, 5 min). Figure 4 shows that Wort pretreatment blocks  $A_1$ R-induced Akt phosphorylation, indicating that PI3K has a piv-



**Figure 2.** The association of A<sub>1</sub> receptor with EGFR in primary cortical neurons. (A) Cells were processed for immunostaining with an Alexa Fluor 488-labeled anti-A<sub>1</sub>R antibody (green) and an Alexa Fluor 555-labeled anti-EGFR antibody (red). (B) Primary cultured cortical neurons were treated with either vehicle or 1 μmol/L CPA for 15 min, and whole cell extracts were immunoprecipitated with an anti-A<sub>1</sub>R antibody or corresponding normal IgG and immunoblotted with antibodies to A<sub>1</sub>R or EGFR. (C) Confocal microscope images show that A<sub>1</sub>R and EGFR are co-localized on the membrane of cortical neurons. Purified cortical neurons were double stained with rabbit anti-A<sub>1</sub>R and mouse anti-EGFR antibodies and species-specific secondary antibodies. Nuclei were counterstained with Hoechst 33258.



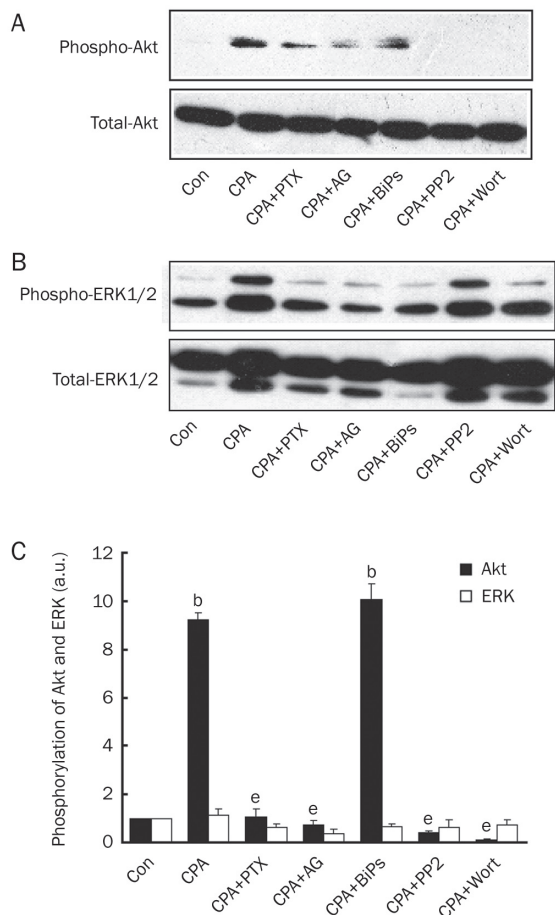
**Figure 3.** The selective EGFR tyrosine kinase inhibitor AG1478 induces a concentration-dependent inhibition of Akt but not ERK1/2 phosphorylation induced by CPA stimulation in primary cortical neurons. Neurons were pretreated with AG1478 at the indicated concentrations for 30 min before stimulation with CPA (100 nmol/L) for 5 min. Cell lysates were analyzed for both phosphorylation of Akt (Ser473) (A) and phosphorylation of ERK1/2 at Thy202/Tyr204 (B) with specific antibodies. Results are representatives of three independent experiments.

otal intermediary role in this cascade.

The major mechanism of EGFR transactivation in response to a variety of external stimuli in numerous cell types is mediated by a soluble EGF-like ligand (HB-EGF), which is generated by the proteolytic processing of the proHB-EGF precursor by metalloproteases; this ligand binds to and activates specific EGFRs<sup>[7, 17]</sup>. Recently, many GPCR agonists have been shown to induce EGFR transactivation through this metalloprotease-dependent HB-EGF shedding<sup>[21]</sup>. In some instances, the intracellular activation of Src kinase has also been implicated in GPCR-mediated EGFR transactivation<sup>[22]</sup>. To evaluate whether the metalloproteases and Src kinase are involved in the transactivation of EGFR via A<sub>1</sub>R, we pretreated cortical neurons with the selective metalloprotease inhibitor, BiPs, or the selective Src kinase inhibitor, PP2, and then stimulated with CPA. PP2 pretreatment attenuated the A<sub>1</sub>R-induced neuroprotection of EGFR (Figure 1F) and activation of Akt (Figure 4A), suggesting that EGFR transactivation during Akt activation depends on Src kinase but not the metalloprotease/HB-EGF shedding in cultured cortical neurons (Figure 6).

#### A<sub>1</sub>R-mediated ERK1/2 activation in HEK293/A<sub>1</sub>R cells involves EGFR transactivation

To confirm the involvement of EGFR transactivation in A<sub>1</sub>R-mediated signaling, we used a HEK293 cell line that is stably



**Figure 4.** Determination of the signaling cascade during Akt activation induced by adenosine  $A_1$  receptor activation. Neurons were pretreated with the indicated inhibitors for 30 min, and then cells were stimulated with vehicle or CPA (100 nmol/L) for 5 min. Cell lysates were analyzed for both phosphorylation of Akt (Ser473) (A) and phosphorylation of ERK1/2 at Thy202/Tyr204 (B) with specific antibodies. The data were quantitated, and the means $\pm$ SEM are shown in the lower panel ( $n=3$ ) (C). <sup>b</sup> $P<0.05$  compared with the untreated control. <sup>e</sup> $P<0.05$  compared with the CPA treated group.

transfected with human  $A_1R$  to determine the signaling cascade that is induced by  $A_1R$  stimulation. The stimulation of HEK293/ $A_1R$  cells with the  $A_1R$  agonist CPA (100 nmol/L) causes rapid but transient ERK1/2 activation. The maximum response in this cell line was achieved by about 5 min (Figure 5A), whereas in cortical neurons, CPA-activated ERK1/2 phosphorylation is sustained for at least 30 min (data not shown). No significant Akt activation was observed using a specific anti-phospho-Akt (Ser473) in these HEK293/ $A_1R$  cells after CPA stimulation (data not shown). We concluded that the ERK1/2 activation by CPA stimulation in HEK293/ $A_1R$  cells was mediated primarily by the transactivation of EGFR, since a pretreatment with AG1478 abolishes the effect of CPA in a concentration-dependent manner (Figure 5B). Unlike in cortical neurons (Figure 2B), the co-immunoprecipitation assay in HEK293/ $A_1R$  cells indicated that  $A_1R$  and EGFR did not form

a complex. We also measured EGFR activation using an antibody against phospho-EGFR/Tyr1608 in HEK293/ $A_1R$  cells and neurons. The phospho-EGFR was detected in HEK293/ $A_1R$  cells but not in cortical neurons.

#### A PTX-sensitive $G_i$ protein acts upstream and PI3K acts downstream of EGFR transactivation during $A_1R$ -mediated ERK1/2 phosphorylation in HEK293/ $A_1R$ cells

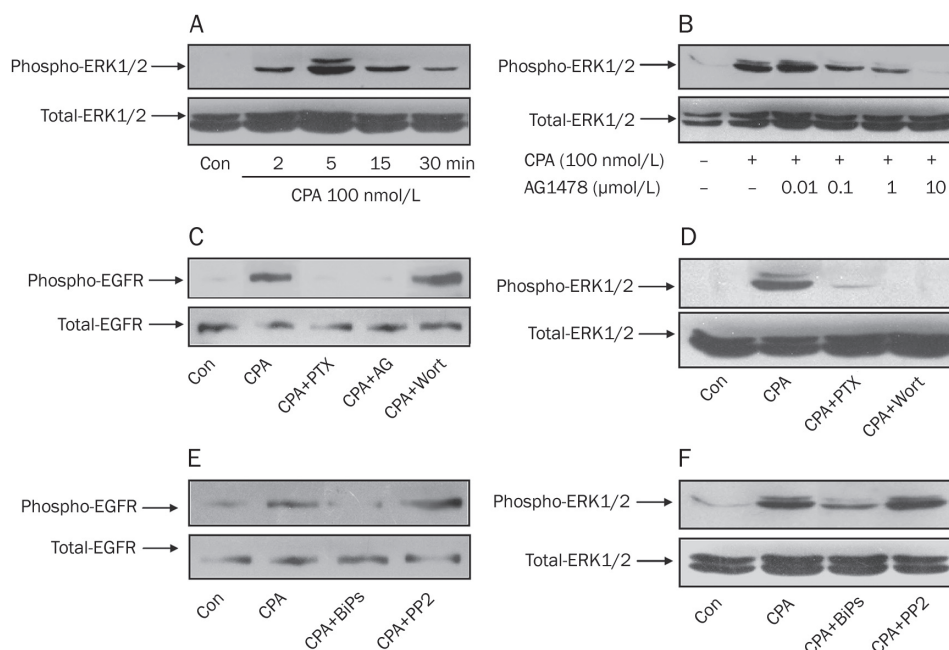
Consistent with the above observations, CPA stimulation can induce the phosphorylation of EGFR in HEK293/ $A_1R$  cells as measured by immunoblotting with a specific anti-phospho-EGFR (Tyr1068) antibody (Figure 5C). It has been reported that a PTX-sensitive  $G_i$  protein is necessary for  $A_1R$ -mediated ERK1/2 activation<sup>[23]</sup>. In our experiments, we found that ERK1/2 was also phosphorylated upon CPA stimulation in HEK293/ $A_1R$  cells via a  $G_i$  protein, since PTX pretreatment (100 ng/mL, 16 h) completely inhibited ERK1/2 activation (Figure 5D). To determine the extent to which PI3K participates in  $A_1R$  action in HEK293/ $A_1R$  cells, we pretreated HEK293/ $A_1R$  cells with wortmannin (Wort, 100 nmol/L) and then stimulated them with CPA (100 nmol/L, 5 min). Figure 5D shows that Wort pretreatment blocked  $A_1R$ -induced ERK1/2 phosphorylation. To determine whether  $G_i$  protein and PI3K act upstream or downstream of EGFR, we measured CPA-induced tyrosine phosphorylation of EGFR following pretreatment with PTX or Wort. Pretreatment with PTX completely blocked EGFR phosphorylation at Tyr1068; this result is consistent with the results of the pretreatment with the EGFR kinase-specific inhibitor, AG1478 (Figure 5C). However, Wort pretreatment had no effect on  $A_1R$ -induced EGFR phosphorylation at Tyr1068. These results indicate that the  $G_i$  protein acts upstream of EGFR, whereas PI3K acts downstream of EGFR.

#### EGFR transactivation in HEK293/ $A_1R$ cells is metalloprotease-dependent and Src kinase-independent

We have demonstrated that EGFR transactivation induced by  $A_1R$  in primary cultured cortical neurons is Src kinase-dependent but metalloprotease/HB-EGF shedding-independent. To evaluate whether the metalloproteases and Src kinase are involved in EGFR transactivation in HEK293/ $A_1R$  cells, cells were pretreated with BiPs or PP2 and then stimulated with CPA. As shown in Figure 5, pretreatment with BiPs attenuates  $A_1R$ -induced activation of ERK1/2 (Figure 5F) and phosphorylation of EGFR at Tyr1068, a Grb2-binding site, in HEK293/ $A_1R$  cells (Figure 5E). These findings demonstrate that ERK1/2 activation depends on metalloprotease action upstream of EGFR in these cells. However, inhibition of Src kinase had no effect on either EGFR phosphorylation (Figure 5E) or ERK1/2 activation (Figure 5F) in HEK293/ $A_1R$  cells, suggesting that  $A_1R$  does not use Src kinase to phosphorylate EGFR and subsequently to induce ERK1/2 activation.

#### Discussion

We have delineated an  $A_1R$ -stimulated signaling pathway that mediates neuroprotection by the specific  $A_1R$  agonist CPA in



**Figure 5.** Effect of EGFR transactivation on the transient activation of ERK1/2 that is induced by  $A_1R$  stimulation in HEK293/ $A_1R$  cells. (A) Time course of the effect of CPA on ERK1/2 phosphorylation at Thy202/Tyr204 in HEK293/ $A_1R$  cells. The cells were stimulated with CPA (100 nmol/L) for the time periods indicated, washed and lysed. The cell lysates were analyzed for phosphorylation of ERK1/2 using a specific antibody. (B) Concentration-dependent inhibitory effects of the selective EGFR tyrosine kinase inhibitor, AG1478, on ERK1/2 phosphorylation induced by CPA stimulation in HEK293/ $A_1R$  cells. The cells were pretreated with AG1478 at the indicated concentrations for 30 min before stimulation with CPA (100 nmol/L) for 5 min. Con indicates untreated control cells. (C–F) Effects of PTX, wortmannin (Wort), BiPs and PP2 on ERK1/2 and EGFR phosphorylation induced by CPA. The cells were stimulated with 100 nmol/L CPA for 5 min and lysed. ERK1/2 (D, F) and EGFR (C, E) phosphorylation was assayed by immunoblotting with an anti-phospho-EGFR (Tyr1068) antibody or an anti-phospho-ERK1/2 (Thy202/Tyr204) antibody, respectively. Then the blots were reprobed with respective total antibodies. Where indicated, cells were pretreated with 100 ng/mL PTX for 16 h, 100 nmol/L Wort, 10 μmol/L BiPs or 5 μmol/L PP2 for 30 min. Results are representatives of three independent experiments.

primary cultured cortical neurons. We also characterized the  $A_1R$ -induced signaling cascade in transfected HEK293/ $A_1R$  cells. Our results show that the EGFR transactivation induced by CPA plays a pivotal role in both cortical neurons that endogenously express  $A_1R$  and in HEK293/ $A_1R$  cells that are exogenously transfected with  $A_1R$ . CPA stimulates the PI3K/Akt pathway through Src-kinase with EGFR transactivation in primary cultured cortical neurons. In HEK293/ $A_1R$  cells, CPA induces a PTX-sensitive, metalloprotease-dependent EGFR phosphorylation with subsequent activation of ERK1/2 but not Akt.

Several lines of evidence indicate that adenosine may be an endogenous neuroprotective agent in the CNS, since it prevents the damage caused by ischemia, excitotoxicity, and epileptic seizures<sup>[24]</sup>.  $A_1R$  agonists have been conclusively shown to attenuate ischemic or excitotoxic neuronal damage, both *in vitro* (cell culture and brain slice) and *in vivo*, in different models of ischemia/hypoxia<sup>[25]</sup>. The acting  $A_1R$  agonists are known to mediate neuroprotection, mostly by blocking  $Ca^{2+}$  influx, which results in the inhibition of glutamate release at the presynaptic level and the reduction of its excitatory effects at a postsynaptic level; they also activate  $K^+$  channels and increase the efflux of  $K^+$ , which leads to hyperpolarization of postsynaptic neurons<sup>[1]</sup>. Although the pharmacological effects

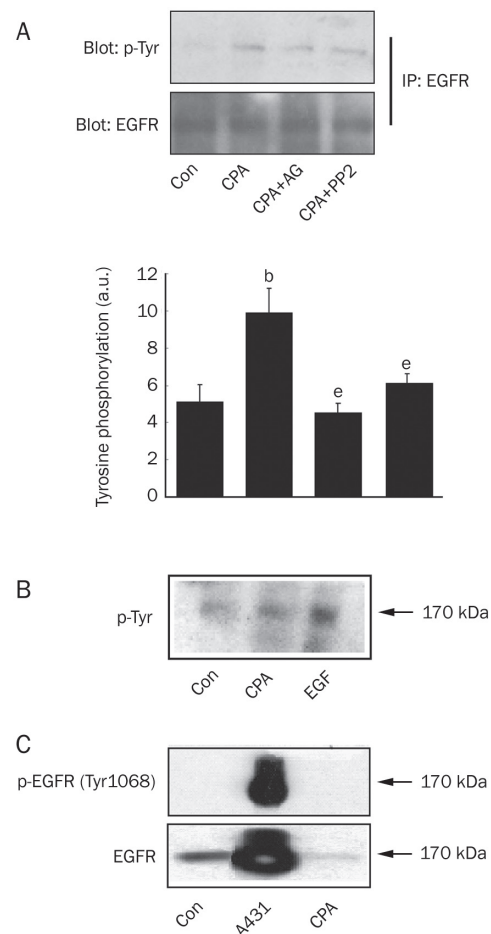
of  $A_1R$  have been extensively studied, the precise signaling pathway involved has remained unclear. Akt is an important regulator of various physiological processes, including glucose metabolism and apoptosis<sup>[26]</sup>, and our data show that  $A_1R$ -mediated PI3K/Akt activation through EGFR transactivation is necessary for the neuroprotective effects using a neuronal OGD model. The present work indicates that EGFR transactivation following  $A_1R$  stimulation underlies, at least in part, the neuroprotective properties.

Many receptor tyrosine kinases (RTKs) undergo transactivation by agonists of GPCRs<sup>[7, 27]</sup>. We observed, in both cortical neurons and HEK293/ $A_1R$  cells, that  $A_1R$  activation can lead to EGFR transactivation. To our knowledge, it has not been reported previously that there is an  $A_1R$ -mediated EGFR transactivation and a physical interaction between  $A_1R$  and EGFR in cortical neurons. The co-localization of the receptors might allow for dynamic, local changes in EGFR activation as a function of adenosinergic neurotransmission.

We show that CPA-activated Akt activation in primary cultured neurons and the transient activation of ERK1/2 in HEK293/ $A_1R$  cells occurs via EGFR transactivation, although sustained ERK1/2 activation is not mediated by EGFR transactivation. Our data are similar to those published in a previous report<sup>[28]</sup>, in which GnRH was shown to induce transient

ERK1/2 activation through EGFR transactivation in GT1-7 cells, but the sustained activation of ERK1/2 in HEK293 cells was shown to occur without transactivation of EGFR. In the case of growth factor-induced ERK1/2 activation, it is known that the magnitude and longevity of the activation depend on the nature of the signaling cascades that are elicited by the stimulation of different growth factors<sup>[29]</sup>. We speculate that in our experiments, at least two signaling cascades were activated. One was triggered by A<sub>1</sub>R stimulation and resulted in EGFR transactivation and Akt phosphorylation in primary cortical neurons and transient ERK1/2 in HEK293/A<sub>1</sub>R cells. Another signaling mechanism simultaneously evoked by CPA stimulation in primary cortical neurons initiated and maintained the sustained ERK1/2 phosphorylation without activating EGFR. ERK1/2 activation, in particular that mediated by RTKs, is closely regulated through multiple forms of negative feedback control mechanisms. The short duration of A<sub>1</sub>R-induced ERK1/2 signaling might be attributable to rapid internalization and degradation of EGFRs<sup>[29]</sup>; also, there is negative feedback phosphorylation of Sos by a kinase downstream of MEK that results in dissociation of the Shc-Grb2-Sos complex<sup>[29]</sup>. Taken together, it is likely that the involvement of EGFR transactivation in A<sub>1</sub>R-induced ERK1/2 activation relies on the engagement of some distinct signaling proteins with the consequence of a transient phosphorylation of ERK1/2.

GPCR-induced activation of EGFR signaling is designated as ligand-independent tyrosine phosphorylation of EGFR<sup>[30]</sup>; EGFR was considered just one of the intermediate signaling proteins<sup>[11]</sup>. Following EGFR phosphorylation, the signaling sequences during GPCR-mediated and EGF-stimulated ERK1/2 and/or Akt activation are generally similar, but the RTKs are phosphorylated at different sites and the consequences of this phosphorylation are distinct. In general, EGFR may be phosphorylated in two ways: in a ligand-dependent manner (eg, EGF and HB-EGF) and in a Src kinase-dependent manner. The former has been reported to include phosphorylation at the residues Tyr992, Tyr1045, and Tyr1068, whereas the latter involves Tyr845<sup>[31]</sup>. In the present study, we found that EGFR transactivation during Akt activation in primary cortical neurons and ERK1/2 activation in HEK293/A<sub>1</sub>R cells were mediated by Src-dependent and HB-EGF shedding-dependent EGFR phosphorylation, respectively. Unlike in HEK293/A<sub>1</sub>R cells, no EGFR phosphorylation at the residue Tyr1068 was detected following A<sub>1</sub>R stimulation in primary cortical neurons (Figure 6C). Src-kinase has been reported to be involved in EGFR phosphorylation and to form a multiprotein complex with GPCR and EGFR<sup>[14]</sup>. The inhibition of either Src or EGFR kinase impairs the ability of CPA to induce the phosphorylation of EGFR and Akt in cortical neurons, indicating that the activation of both proteins is required for this cascade. Moreover, inhibiting either Src or EGFR completely abolishes the ability of CPA to preserve cell viability during OGD insult. Therefore, we propose that, in the immunocomplex (possibly as part of a larger multiprotein complex), A<sub>1</sub>R transactivates EGFR through Src kinase, which in turn activates the cytoprotective PI3K/Akt pathway in cortical



**Figure 6.** Effects of CPA on EGFR phosphorylation in primary cortical neurons. Neurons were pretreated with or without 1  $\mu$ mol/L AG1478 or 5  $\mu$ mol/L PP2 for 30 min and then stimulated with 1  $\mu$ mol/L CPA for 15 min. Cells were lysed and split into two groups. One was prepared for immunoblotting with an anti-phospho-tyrosine (p-Tyr) antibody (B) or a specific anti-phospho-EGFR (Tyr1068) antibody (C). A431 substrate stands for lysates of A431 cells stimulated with EGF as a positive control. The other group was immunoprecipitated with an anti-EGFR antibody. Immunoprecipitates were assayed by immunoblotting with an anti-phosphotyrosine (p-Tyr) antibody (A). The p-Tyr blots were then stripped and reprobbed with an anti-EGFR antibody. The quantitated data are shown as the means $\pm$ SEM. ( $n=3$ ). <sup>b</sup> $P<0.05$  compared with the untreated control group, and <sup>e</sup> $P<0.05$  compared with the CPA treated group. Notably, probing with the anti-p-Tyr antibody revealed EGFR tyrosine phosphorylation following CPA stimulation and the reduction in A<sub>1</sub>R-mediated EGFR tyrosine phosphorylation upon AG1478 or PP2 pretreatment. Unlike in HEK293/A<sub>1</sub> cells, in primary cortical neurons CPA stimulation does not induce EGFR phosphorylation at Tyr1068 (C).

neurons. In HEK293/A<sub>1</sub>R cells, however, A<sub>1</sub>R-mediated EGFR transactivation is Src-independent but EGFR ligand-dependent (eg, EGF and HB-EGF). The lack of a complex between A<sub>1</sub>R and EGFR in these cells may be due to the involvement of the metalloprotease-mediated HB-EGF shedding pathway in this mechanism.

EGFR activation leads to the recruitment and activation of



other signaling proteins, such as phospholipase C $\gamma$  and PI3K, and/or adaptor proteins, such as Shc, Grb2, and Sos. PI3K is an important signaling molecule during cell survival and is activated by EGFR phosphorylation. A substantial amount of data also supports a crucial role for PI3K in GPCR signaling during proliferation and growth. GPCR-mediated activation of ERK1/2 has been reported to be dependent<sup>[32]</sup> or independent<sup>[33]</sup> of PI3K activation. We used Wort to examine the effect of PI3K inhibition on A<sub>1</sub>R-mediated signaling in both cell types in which Akt activation (primary cortical neurons) and ERK1/2 activation (HEK293/A<sub>1</sub>R cells) depend on EGFR transactivation. Our results show that both Akt activation (primary cortical neurons) and ERK1/2 activation (HEK293/A<sub>1</sub>R cells) are mediated by PI3K.

In conclusion, we show that an A<sub>1</sub>R agonist can activate the Src/EGFR/PI3K/Akt pathway to mediate neuroprotection in cortical neurons. A<sub>1</sub>R is often associated with cellular responses to ischemic and metabolic stress. Previous results<sup>[34]</sup> have shown that A<sub>1</sub>R accumulates in neurodegenerative brain tissues in Alzheimer disease and mediates both amyloid precursor protein processing and Tau phosphorylation and translocation. Therefore, further in-depth study of the transactivation and regulation of EGFR by A<sub>1</sub>R, in particular the neuroprotective and synergistic action of their ligands, would be important for the development of novel therapeutic strategies and drugs effective against neurodegenerative diseases.

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### Author contribution

Lin-yin FENG, Ke-qiang XIE designed the research; Ke-qiang XIE, Li-min ZHANG, Yan CAO, and Jun ZHU performed the research; Ke-qiang XIE and Li-min ZHANG analyzed the data; Lin-yin FENG and Ke-qiang XIE wrote the paper.

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