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# μ Opioid Transactivation and Down-Regulation of the Epidermal Growth Factor Receptor in Astrocytes: Implications for Mitogen-Activated Protein Kinase Signaling

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## ABSTRACT

Astroglia are a principal target of long-term μ antiproliferative actions. The mitogen-activated protein (MAP) kinase known as extracellular signal-regulated kinase (ERK), is a key mediator of cell proliferation. In studies on the mechanism of short- and long-term μ opioid regulation of the ERK signaling pathway, we show that the μ opioid agonist [D-Ala$^2$,*N*-Me-Phe$^4$,Gly$^5$-ol]-enkephalin (DAMGO), acting via the endogenous μ opioid receptor (MOR), induced sequential epidermal growth factor receptor (EGF) receptor (EGFR) Tyr phosphorylation, Ser phosphorylation, and down-regulation in immortalized rat cortical astrocytes. The short-term action of DAMGO resulted in the stimulation of ERK phosphorylation. 4(3-Chlorophenylamino)-6,7-dimethoxyquinazoline (Tyrphostin AG1478), a selective inhibitor of EGFR Tyr kinase activity, blocked EGFR and ERK activation by short-term DAMGO administration, implicating EGFR transactivation in its stimulation of ERK activity. Inhibitors of matrix metalloproteinases attenuated MOR-mediated ERK phosphorylation, suggesting that shedding of EGF-like ligands from the plasma membrane may be involved in the EGFR transactivation process. Prolonged DAMGO exposure induced EGFR internalization/down-regulation, did not activate ERK, and inhibited exogenous EGF-stimulated ERK phosphorylation. MOR-mediated EGFR down-regulation seems to be MAP kinase-dependent, because it was inhibited by the ERK kinase inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126), and tyrphostin AG1478. The κ opioid agonist (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl) benzeneacetamide (U69,593) induced Tyr and Ser phosphorylation of EGFR and activation of ERK. However, long-term application of U69,593 neither down-regulated EGFR nor inhibited EGF-induced ERK activation. Instead, it engendered a sustained activation of ERK. Collectively, our data suggest that long-term application of DAMGO initiates heterologous down-regulation of EGFR via a mechanism involving ERK in astrocytes.

GPCR signaling to ERK can vary considerably, and cell-type specificity is one of the determinants of this diversity. Thus, GPCR and growth factor receptor signaling can converge at several different points to achieve activation of the MAP kinase known as ERK. It has been reported that GPCR signaling to ERK can occur via endogenous agonist-induced Tyr phosphorylation of the growth factor receptor itself (Daub et al., 1996; Eguchi et al., 1996; Belcheva et al., 2001). This mechanism is cell-type specific. For example, lysophosphatidic acid stimulation of ERK phosphorylation is EGFR transactivation-dependent in Rat-1 but not PC-12 cells (Della Rocca et al., 1999). MOR mediates EGFR transactivation-independent and dependent signaling to ERK in human embryonic kidney 293 cells (Belcheva et al., 2001). The EGFR transactivation-dependent pathway required calmodulin binding to MOR.

RTK transactivation seems to occur via membrane-bound metalloproteinase (matrix metalloproteinases/metalloproteinases with a disintegrin domain), that are involved in processing of EGF-like precursor molecules anchored on the cell surface (Prenzel et al., 1999; Yan et al., 2002). In some pathways, PKC and/or Src may activate metalloproteinases (Pierce et al., 2001). A similar EGFR transactivation mechanism involving an autocrine metalloproteinase-dependent release of heparin-binding EGF resulting from insulin-like growth factor stimulation has been reported (Roudabush et al., 2000). Transactivation of other RTKs occurs via GPCR

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; ERK, extracellular signal-regulated kinase; MAP, mitogen activated protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MEK, extracellular signal-regulated kinase kinase; MOR μ opioid receptor; RTK, receptor tyrosine kinase; PKC, protein kinase C; PDGFR, platelet-derived growth factor receptor; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; DAMGO, [D-Ala$^2$,*N*-Me-Phe$^4$,Gly$^5$-ol]-enkephalin; CTAP, D-Phe-Cys-Trp-Arg-Thr-Pen-Thr-$NH_2$; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl) benzeneacetamide; nor-BNI, nor-binalthorphimine; DMEM, Dulbecco's modified Eagle's medium; KOR, κ opioid receptor; TBST, Tris-buffered saline + 0.2% Tween 20; HRP, horseradish peroxidase; tyrphostin AG1478, 4(3-chlorophenyamino)-6,7-dimethoxyquinazoline; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; PTX, pertussis toxin.

signaling. These include angiotensin-induced PDGFR Tyr phosphorylation (Linseman et al., 1995) in vascular smooth muscle cells, lysophosphatidic acid-stimulated PDGFR Tyr phosphorylation in L cells by a PKC-independent mechanism (Herrlich et al., 1998), and $\mu$ opioid-stimulated FGFR phosphorylation in rat C6 glioma cells (Belcheva et al., 2002). In these studies, blockade of RTK Tyr phosphorylation results in attenuation of opioid-induced ERK phosphorylation. The mechanism of transactivation of PDGFR and FGFR has not been characterized to the extent that it has been for EGFR, although evidence for a metalloproteinase requirement has been obtained in $\mu$ and $\kappa$ opioid-induced ERK phosphorylation studies in C6 cells (Belcheva et al., 2002).

The integral role of RTKs in short-term $\mu$ and $\kappa$ opioid agonist-induced activation of ERK raises the question of their possible involvement in the negative regulation of ERK by long-term application of opioid agonists. Treatment with $\mu$ or $\kappa$ opioid agonists for 2 h or longer attenuated exogenous EGF-induced ERK activation in OR overexpressing COS-7 cells (Belcheva et al., 1998). Although short-term administration of $\mu$ and $\kappa$ opioids activate ERK via a PTX-sensitive G protein, PTX insensitivity of the long-term $\mu$ (but not $\kappa$) action was observed. Furthermore, the PTX-insensitive G proteins $G_Z$ and $G_{12}$, can transduce long-term $\mu$ but not $\kappa$ opioid inhibition of EGF-stimulated ERK in COS-7 cells, suggesting differences in the two opioid pathways (Belcheva et al., 2000). Different signal transduction mechanisms mediated by endogenous KOR and MOR were also observed in C6 cells grown under conditions in which they express an astrocytic phenotype (Bohn et al., 2000a,b). Long-term administration of $\mu$ agonists inhibited short-term $\kappa$ opioid agonist- and endothelin-stimulated ERK phosphorylation and DNA synthesis. However, long-term administration of $\kappa$ opioid had no effect on mitogen-stimulated ERK phosphorylation.

To examine the role of RTKs in the mechanism of regulation of ERK phosphorylation by opioids, we chose astrocytes as our model system. Astrocytes are known to express $\mu$, $\delta$, and $\kappa$ opioid receptors and to respond to opioid agonists by decreasing or increasing proliferation in vivo and in vitro (Eriksson et al., 1991; Stiene-Martin and Hauser, 1991; Ruzicka et al., 1995; Stiene-Martin et al., 2001). Astrocytes also express EGF, FGF, and their receptors (Finklestein et al., 1988; Nieto-Sampedro et al., 1988; Kornblum et al., 1998). When morphine, a $\mu$ agonist, is administered to mice, it significantly reduces the number of striatal BrdU-labeled GFAP(+) cells compared with controls (Stiene-Martin et al., 2001). Long-term administration of morphine to rats enhanced levels of GFAP and phosphorylated ERK in the ventral tegmental area (Beitner-Johnson et al., 1993; Berhow et al., 1996). In addition, antiproliferative actions of morphine in cerebellar neuroblasts were reversed by heparin-EGF, a form of the growth factor that enhanced proliferation of neuroblasts and has been implicated in GPCR-induced EGFR transactivation that results in ERK activation (Opanashuk and Hauser, 1998). EGF and FGF are essential growth factors for astrocyte development in perinatal brain and in adult neural progenitor cell differentiation to astrocytes (Kornblum et al., 1998; Learish et al., 2000). Both growth factors promote proliferation of adult neural stem cells (Learish et al., 2000; Doetsch et al., 2002). Moreover, adult neural progenitor cells may have an astrocytic lineage, and astrocytes play an important role in neurogenesis in adult brain (Doetsch et al., 1999, 2002; Song et al., 2002). Opioids regulate the proliferation of EGF- and FGF-regulated adult neural progenitor cells via MOR-mediated ERK activation (Persson et al., 2003) and long-term morphine inhibits their neurogenesis in vivo (Eisch et al., 2000).

Here we correlated opioid regulation of EGFR activation and down-regulation with ERK phosphorylation in rat astrocytes. The focus of this study is the mechanism of action of long-term $\mu$ and $\kappa$ opioid agonists on ERK phosphorylation.

## Materials and Methods

**Reagents.** Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) with the following exceptions: DAMGO trifluoroacetate and CTAP were obtained from Multiple Peptide Systems (San Diego, CA); U69,593 from the National Institute on Drug Abuse Drug Supply (Research Triangle, NC); nor-BNI was from RBI/Sigma (Natick, MA); EGF (human, recombinant) was from Invitrogen (Carlsbad, CA); tyrphostin AG1478 was from Calbiochem (La Jolla, CA); ilomastat from Chemicon (Temecula, CA); Protein G Plus Agarose suspension was from Oncogene Research Products (San Diego, CA); DMEM and fetal bovine serum were from the American Type Culture Collection (Manassas, VA). Anti-phospho ERK antibody and anti-phospho-Tyr antibody (P-Tyr-100, mouse monoclonal) were purchased from Cell Signaling Technology (Beverly, MA); anti-ERK antibody and anti-EGFR antibody (sheep polyclonal) were purchased from Upstate Biotechnology (Lake Placid, NY); and anti-phosphoserine antibody was purchased from Zymed Laboratories (South San Francisco, CA).

**Cell Cultures.** Rat cortical astrocytes (CTX TNA2; American Type Culture Collection), were established from cultures of primary type 1 astrocytes from 1-day-old rat brain frontal cortex. The cultures were originally transfected with a DNA construct containing the oncogenic early region of simian virus 40 under the transcriptional control of human GFAP promoter (Radany et al., 1992). The astrocytes have the phenotypic characteristics of type 1. This cell line was maintained in DMEM + 10% fetal bovine serum. For all experiments, early passage (2–6) cells were grown in six-well plates at least overnight to adhere well to the plate surface, and optimal starvation was achieved in DMEM containing no serum for 24 h. In all assays, agonists, antagonists, or inhibitors were delivered in serum-free media.

**Transient Transfections.** In some cases, cultures were transfected with pcDNA3 (for mock transfections), rat MOR or KOR cDNA (pCMV-neo expression vector) using FuGENE 6 transfection reagent following the manufacturer's instructions and using 1 $\mu$g of cDNA and 3 $\mu$l of transfection reagent. After 24 to 48 h of incubation, transfection media was replaced with serum-free media for an additional 24 h. The efficiency of transfection was determined to be 9 $\pm$ 1% ($n$ = 6) by in situ identification of cells expressing $\beta$-galactosidase. Negative controls included untransfected and mock-transfected cells.

**ERK Assays.** ERK phosphorylation was measured by immunoblotting as described previously (Belcheva et al., 2001). Briefly, cultures were pretreated with different inhibitors, followed by DAMGO, U69,593, or EGF addition as described in the figure legends. Cells were then washed with cold phosphate-buffered saline and lysed with buffer containing 20 mM HEPES, 10 mM EGTA, 40 mM $\beta$-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM sodium vanadate, 1% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu$g/ml aprotinin, and 20 $\mu$g/ml leupeptin. Lysates were spun at 14,000$g$ for 20 min at 4°C and protein concentration of the supernatants was determined. Cell lysates (15–20 $\mu$g protein/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific sites were blocked with 5% milk in Tris-buffered saline +

0.2% Tween 20 (TBST). Blots were then washed three times with TBST and incubated with anti-phospho-ERK antibody diluted 1:2000 in TBST for at least 15 h at 4°C. After three washes with TBST, blots were incubated with 1:2000 diluted goat anti-mouse–HRP-conjugated IgG (Sigma, St. Louis, MO) for 1 h at room temperature. For assurance of equivalent total ERK protein per lane, representative blots were stripped (0.2 M glycine, pH 2.5, 60 min at room temperature) and exposed to antibodies for ERK, followed by goat anti-rabbit-HRP conjugated IgG. Bands were visualized using an ECL chemiluminescence detection system from Amersham Biosciences (Piscataway, NJ) and exposure to Classic Blue sensitive X-ray film (Molecular Technologies, St. Louis, MO). Band intensities were determined by densitometric analysis using a Kodak DC120 1.2-megapixel digital camera, Kodak ds 1D version 3.0.2 software (Kodak Scientific Imaging Systems, New Haven, CT) and Scion Image software (Scion Corp., Frederick, MD).

**EGFR Immunoprecipitation and Immunoblotting.** Cells were serum-starved for 24 h and treated with DAMGO, U69,593 (0.1 μM, 5–10 min), or EGF (0.1 μg/ml, 5 min). In some experiments, cells were pretreated with AG1478 (0.1 μM) and then exposed to opioid agonist (0.1 μM, 5–10 min). Cultures were lysed as described previously (Belcheva et al., 2001) by using a modified radioimmunoprecipitation assay buffer containing: 50 mM Tris-HCl, pH 7.4, 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM $Na_3VO_4$, and 1 mM NaF. Cell lysates of 0.5 to

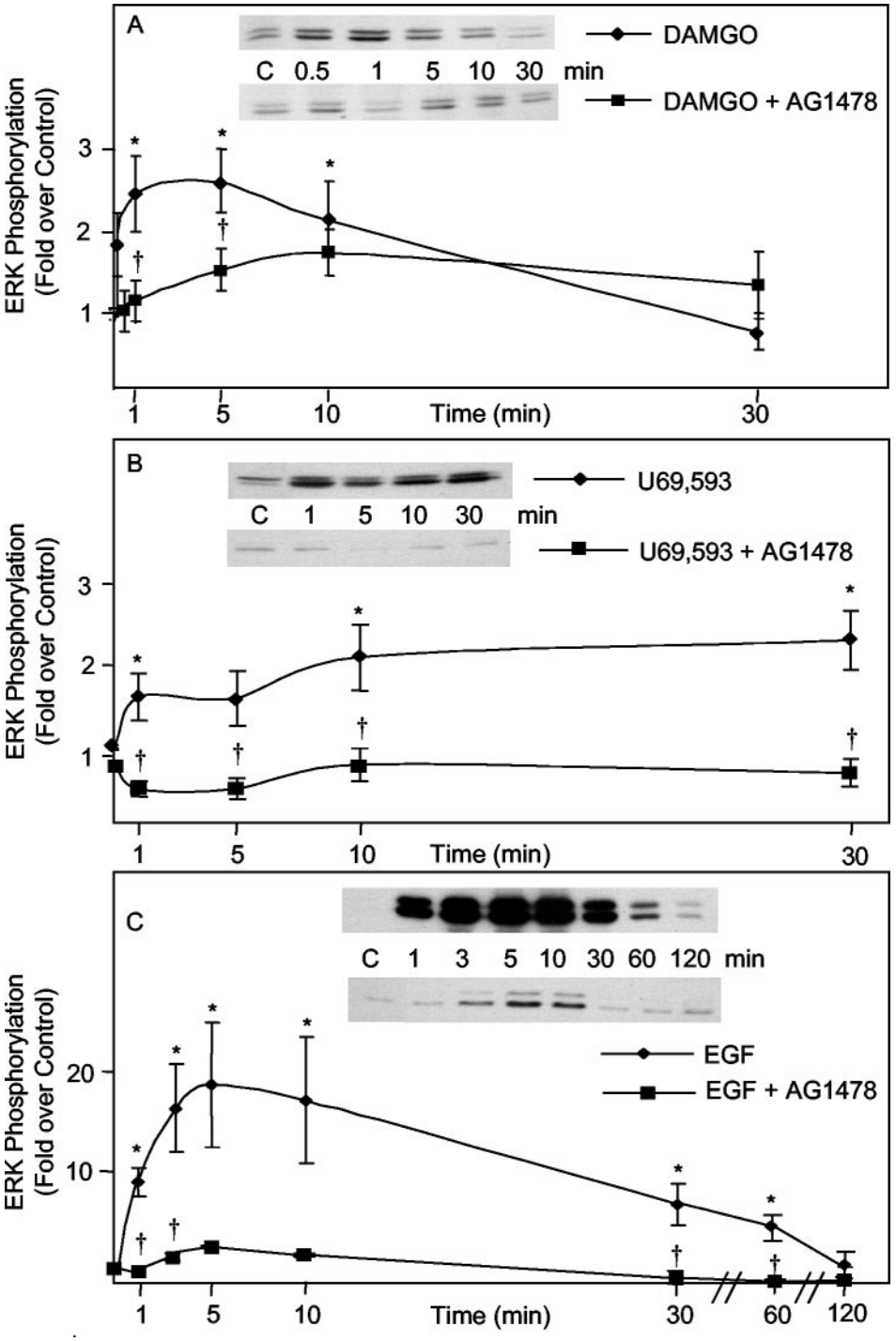


**Fig. 1.** Time course of opioid agonist- and EGF- induced ERK phosphorylation in the presence and absence of tyrphostin AG1478. DAMGO (0.1 μM) (A), U69,593 (0.1 μM) (B), or GF (25–50 ng/ml) (C) were added to rat astrocytes for the indicated time intervals. In some experiments, cells were pretreated with AG1478 (0.1 μM) for 20 min before exposure to the corresponding agonist. Immunoblotting was performed with phospho-ERK antibody. ERK phosphorylation is expressed as -fold over control in the presence or absence of inhibitor. Basal levels of ERK were not changed in the presence of AG1478 alone (1.0 ± 0.1 versus controls) $n = 3–25$. *, $P < 0.05$; significantly greater than controls. †, significantly less than the corresponding agonist alone, .

1.0 mg of protein (diluted to ~1 $\mu$g/$\mu$l) were used. EGFR was immunoprecipitated by adding 5 to 10 $\mu$g of a sheep polyclonal anti-EGFR antibody (Upstate Biotechnology, Lake Placid, NY) to the lysates and incubating overnight at 4°C. This step was followed by addition of a 50-$\mu$l suspension of protein G plus Sepharose beads per sample and incubation for 2 h at 4°C. Beads were washed three times with phosphate-buffered saline, resuspended in SDS loading buffer, and boiled for 5 min before SDS-polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P polyvinylidene difluoride membranes and incubated with 1:2000 dilution of anti *p*-Tyr followed by 1:2000 diluted goat anti-mouse-HRP conjugated IgG. Bands were visualized using a chemiluminescence detection system, as described above.

For direct EGFR immunoblotting experiments, cells were lysed in modified radioimmunoprecipitation assay buffer (Belcheva et al., 2001), and samples containing 20 to 50 $\mu$g of protein were directly loaded on 7.5% SDS gels. After transferring proteins to membranes, detection of the EGFR band (about 170 kDa) was accomplished by applying either a rabbit polyclonal anti-EGFR antibody (Santa Cruz) or a sheep polyclonal anti-EGFR antibody (Upstate Biotechnology). Anti-rabbit or anti-sheep IgG linked to HRP was used as a secondary antibody. Bands were visualized using a chemiluminescence detection system as described above.

**$^{125}$I-EGF Binding to Cell Surface EGFR in Rat Astrocytes.** DAMGO-induced EGFR down-regulation was measured as described with some modifications (Sorkin et al., 1996). Briefly, cells grown in 24 well plates in media without serum for 24 h were treated with either 100 ng/ml EGF for 30 min or with 100 nM DAMGO for different time intervals at 37°C. At the end of the incubation period, cells were rinsed twice with ice-cold media and the plates were placed on ice. Surface bound EGFR ligands were removed by ice-cold acid wash of cells with 0.2 M sodium acetate buffer, pH 4.5, for 2 min, followed by extensive washing with ice-cold media. Remaining cell-surface EGFR binding was determined by incubating cells on ice

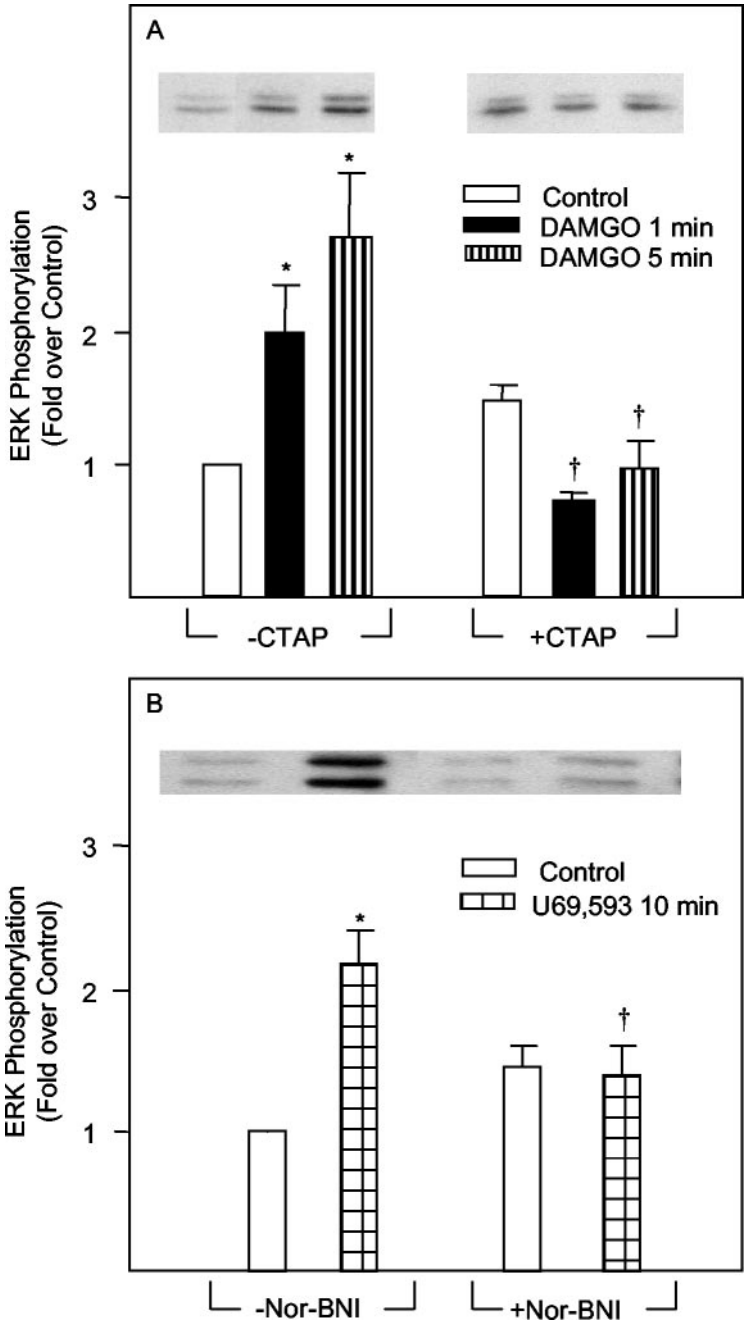


**Fig. 2.** Corresponding opioid antagonists block agonist stimulation of ERK phosphorylation. Astrocytes were treated with (A) DAMGO (0.1 $\mu$M) or (B) U69,593 (0.1 $\mu$M) for 1 to 10 min. In some experiments, cultures were preincubated for 1 h with either CTAP (1 $\mu$M) or nor-BNI (1 $\mu$M) before treatment with the corresponding agonist for 1 to 10 min. $n$ = 3–11 *, significantly different from control; †, $P < 0.05$; significantly less than agonist alone.

with 1 nM $^{125}$I-EGF in binding media, which contained 20 mM HEPES and 0.1% bovine serum albumin, for 2 h. DAMGO (100 nM) was present in the binding media of opioid-treated cells during the 2-h incubation period. This was followed by four washes with ice-cold media to remove unbound ligand. Nonspecific binding was estimated for each time point in the presence of 500 ng/ml EGF. Cells were lysed in 1 N NaOH to measure bound radioactivity by γ counting. For protein determinations, all cell lysates were neutralized with equal amounts of 1 N HCl. Specific binding was calculated as disintegrations per minute per milligram of protein and expressed as percentage of control.

**Statistical Analysis.** Statistical determinations were made by *t* test analysis using Prism software version 2.01 (GraphPad Software, San Diego, CA). Data are expressed as the mean ± S.E.M.

## Results

**Time Course of DAMGO, U69,593, and EGF Stimulation of ERK Phosphorylation in Rat Astrocytes.** Both μ- and κ-specific agonists stimulated phosphorylation of ERK in a time- (Fig. 1) and concentration-dependent manner. It is noteworthy that μ and κ opioids display different time courses in stimulating ERK phosphorylation. DAMGO induced a rapid, short lasting activation of ERK, whereas the κ agonist U69,593 elicited a sustained activation of ERK for 30 min and longer (see below). Dose dependence studies of DAMGO and U69,593 stimulation of ERK phosphorylation gave typical saturation plots with optimal effects in the nanomolar range (data not shown). U69,593 stimulation of ERK phosphorylation was more potent than DAMGO.

Previous immunoblotting data suggested that EGFR is relatively abundant in the rat cortical astrocytes used in this study (Belcheva et al., 2002). The time dependence of EGF-stimulated ERK phosphorylation was compared with that of μ and κ opioids. As shown in Fig. 1C, EGF induced a rapid stimulation of ERK with optimal values at 3 to 10 min. A substantial decline in ERK phosphorylation seems to occur by 30 to 60 min of EGF treatment, comparable with that seen in the DAMGO time course but unlike the sustained stimulation by U69,593. These results suggest that homologous EGFR desensitization occurs within 30 to 60 min of EGF exposure.

MOR antagonist CTAP or KOR antagonist nor-BNI blocked DAMGO or U69,593 stimulation of ERK phosphorylation, respectively (Fig. 2). Antagonists alone did not affect ERK phosphorylation. These results suggest that μ and κ opioid stimulation of ERK phosphorylation is mediated by endogenous MOR and KOR, respectively in rat astrocytes.

**EGFR Transactivation Is a Required Step in MOR and KOR Stimulation of ERK Phosphorylation.** To investigate a possible role of EGFR transactivation in opioid stimulation of ERK phosphorylation, astrocytes were treated with the selective EGFR kinase inhibitor tyrphostin AG1478 followed by DAMGO or U69,593 treatment for different time intervals and then either ERK phosphorylation (Fig. 1, A and B) or Tyr phosphorylation of EGFR (Fig. 3) was measured. As a positive control in the EGFR phosphorylation assay, cells were also treated with exogenous EGF. AG1478 alone did not attenuate basal levels of ERK phosphorylation or Tyr phosphorylation of EGFR, but it abolished DAMGO-, U69,593- and EGF-induced ERK phosphorylation and Tyr phosphorylation of EGFR, suggesting that both MOR and KOR activation of ERK requires an EGFR transactivation step. Upon transient transfection of rat astrocytes with KOR cDNA, the extent of induction of EGFR phosphorylation by U69,593 was similar to that elicited by EGF (Fig. 3). When astrocytes containing endogenous KOR were used to measure levels of EGFR phosphorylation, the stimulation was 1.7 ± 0.4, 1.2 ± 0.2 and 1.6 ± 0.2 fold higher than basal values ($n = 4$) after U69,593 treatment for 0.5, 1, and 2 min, respectively.

**Metalloproteinase Inhibitors Attenuate μ and κ Opioid Stimulation of ERK Phosphorylation.** Because metalloproteinases are involved in the shedding of endogenous EGF-like ligands from their plasma membrane anchor during transactivation, metalloproteinase inhibitors can provide additional evidence to implicate EGFR activation. When astrocytes were pretreated with *o*-phenanthroline or phosphoramidon, μ and κ agonist stimulation of ERK phosphorylation were significantly reduced by both of these more general

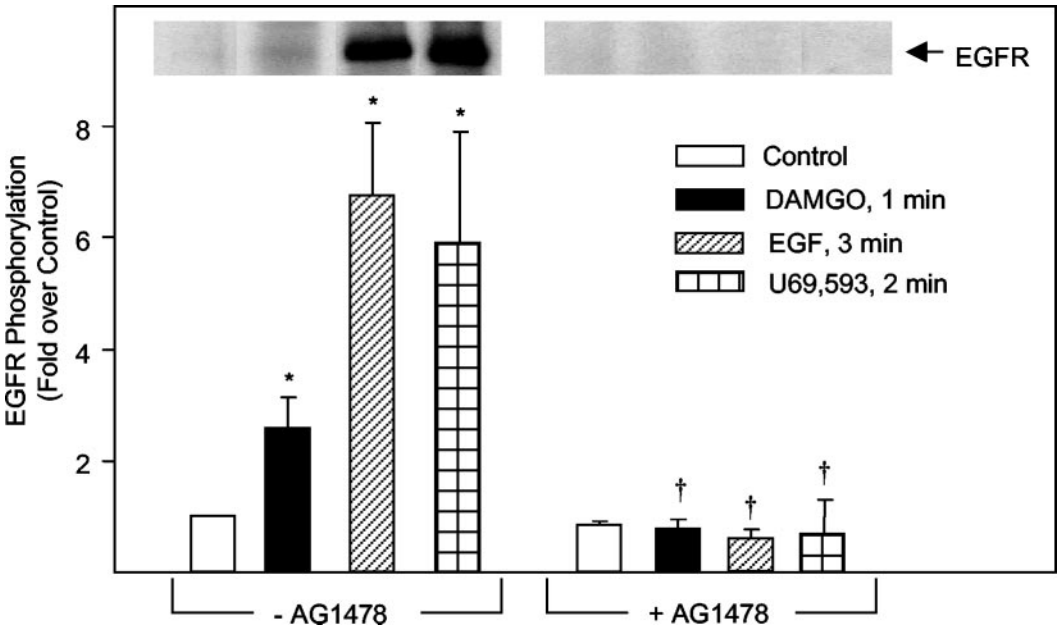


**Fig. 3.** Opioid stimulation of EGFR phosphorylation. Astrocytes were treated with DAMGO (0.1 μM, 1 min), U69,593 (0.1 μM, 2 min), or EGF (0.1 μg/ml, 3 min). In some experiments, cells were pretreated with AG1478 (0.1 μM) for 20 min before exposure to opioids. EGFR was immunoprecipitated with an anti-EGFR antibody and immunoblotting was performed with a phospho-Tyr antibody. Endogenous MOR activation of EGFR was measured, whereas transiently transfected KOR astrocytes were used for U69,593 activation of EGFR. $n = 3$–6. *, significantly more than control; †, $P < 0.05$; significantly less than agonist alone, .

metalloproteinase inhibitors (Fig. 4A). In addition, ilomastat, a more specific hydroxamic acid-based matrix metalloproteinase/metalloproteinase with a disintegrin domain inhibitor, attenuated MOR and KOR stimulation of ERK phosphorylation. When astrocytes were treated with exogenous EGF or basic FGF, phosphoramidon and ilomastat slightly (19–30%) reduced ERK phosphorylation (Fig. 4B). The metalloproteinases may block a small amount of EGFR transactivation via secreted endogenous EGF (Roudabush et al., 2000).

**Long-Term Treatment with DAMGO, but Not U69,593, Inhibits EGF-Stimulated ERK Phosphorylation.** In our original time course studies (Fig. 1), we observed that DAMGO stimulation of ERK phosphorylation subsided after 30 min, whereas that of U69,593 persisted. In Fig. 5A, we show that this effect of U69,593 on ERK phosphorylation observed at 30 min was sustained for at least an additional 2 h, whereas long-term DAMGO had no effect on ERK phosphorylation at this time interval. We also studied the effects of long-term opioids on EGF-stimulation of ERK phosphorylation. Treatment of rat astrocytes with DAMGO for 2 h diminished EGF-stimulated ERK phosphorylation (Fig. 5B). In contrast, U69,593 failed to attenuate EGF-stimulated ERK phosphorylation after 2 h of exposure (Fig. 5B).

**Long-Term Treatment with DAMGO, but Not U69,593, Down-Regulates EGFR.** ERK phosphorylation by DAMGO or U69,593 occurs within minutes via EGFR transactivation (Figs. 1 and 3). However, the activation of ERK by the $\kappa$ agonist is sustained, whereas that induced by

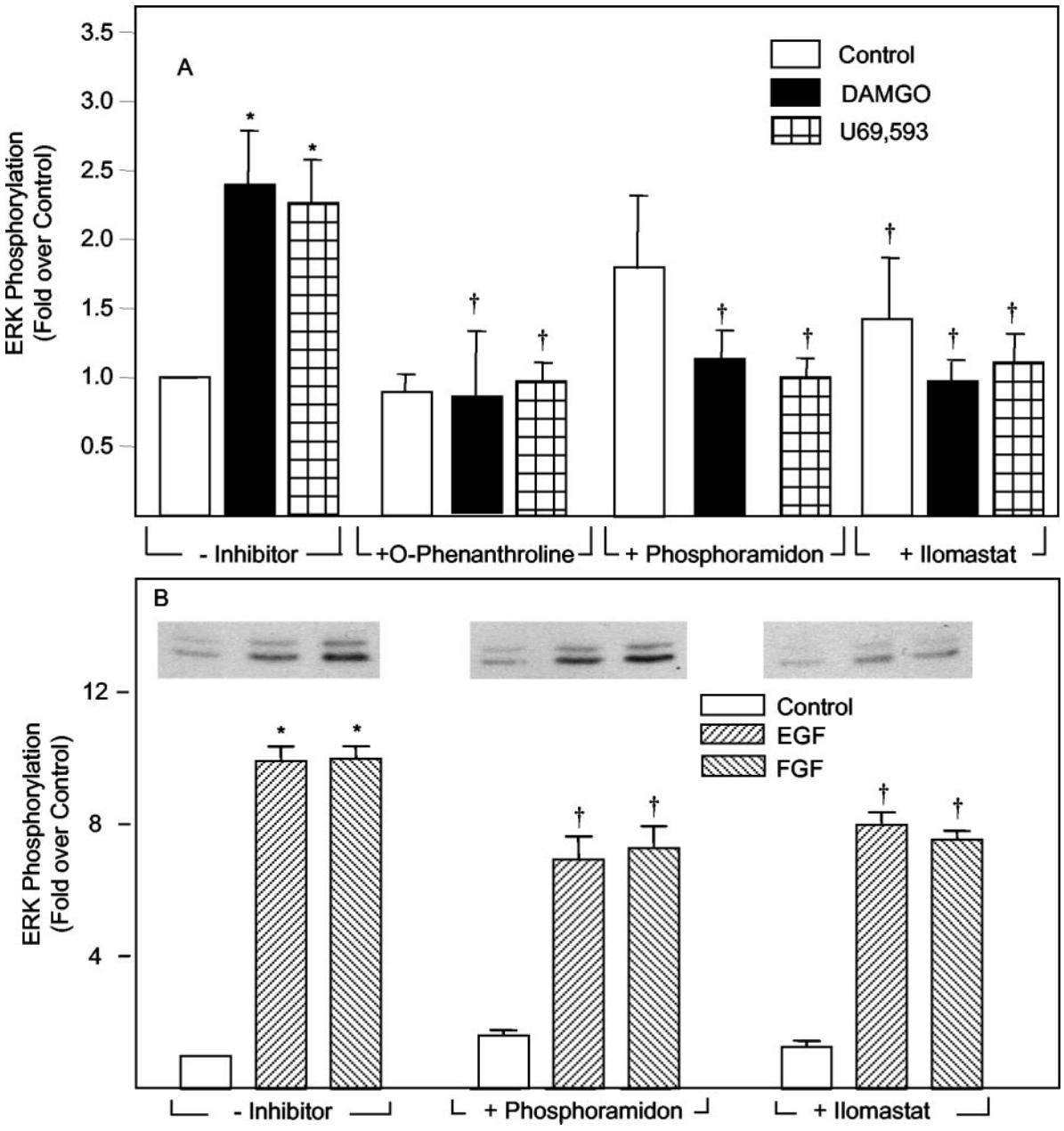


**Fig. 4.** Metalloproteinase inhibitors abolish MOR and KOR stimulation of ERK phosphorylation. Astrocytes were pretreated with either *o*-phenanthroline (200 $\mu$M), phosphoramidon (300 $\mu$M) or ilomastat (2 $\mu$M) for 1h before exposure to DAMGO (0.1 $\mu$M, 5–10 min) and U69,593 (0.1 $\mu$M, 10 min) (A) or EGF or basic FGF (100 ng/ml, 3 min) (B). $n$ = 4–20 experiments. *, significantly more than controls. †, $P < 0.05$; significantly less than opioid agonist or growth factor alone.

the long-term μ agonist returns to basal levels within 30 min. Because the effect of the μ agonist may be explained by heterologous desensitization of EGFR, the approach taken here was to determine the changes induced by long-term DAMGO or U69,593 on EGFR levels and binding activity. Cells were treated with DAMGO or U69,593 for different time intervals before measuring changes in EGFR levels by cell surface EGFR binding assays and immunoblotting. Upon determining $^{125}$I-EGF binding to EGFR on intact cells, we found that DAMGO pretreatment of cells induced a 30% reduction in cell surface binding relatively rapidly (15 min; Fig. 6A). In contrast, EGF (100 ng/ml, 30 min) diminished cell surface EGFR binding by 90%. As shown in Fig. 6B, DAMGO (2 h) induced a 30% reduction in the EGFR levels. This occurs after the internalization of cell surface receptors (30 min). In comparison, EGF elicited a 40 to 60% decrease in EGFR content and in ERK phosphorylation by 30 min (Figs. 1C and 6, B and C). Pretreatment of cells with AG1478 abolished DAMGO (2 h)-induced reduction in EGFR levels (Fig. 6B). AG1478 alone did not change basal EGFR levels. In fact, the combination of AG1478 and DAMGO up-regulated EGFR levels by 59%. Up-regulation of EGFR may be caused by the blockade of the EGFR Tyr phosphorylation and internalization.

Although transactivation of EGFR was initiated within 1 to 5 min of DAMGO exposure (Fig. 3), levels of cell surface binding of this RTK remained unchanged in this time interval (Fig. 6). In contrast to DAMGO, long-term U69,593 failed to down-regulate EGFR after 120 min (Fig. 6C), consistent with its sustained ERK phosphorylation (Fig. 1B). In fact, EGFR levels remained unchanged for as long as 4 h of exposure to 0.1 μM U69,593 in rat astrocytes overexpressing KOR (1.3 ± 0.2-fold over controls, $n = 4$).

Phosphorylation of Ser residues on EGFR has been shown to be associated with the desensitization process of this receptor. Thus, we conducted experiments to assess the effect of μ and κ opioids on EGFR Ser phosphorylation. As seen in Fig. 7A, short-term application (5 min) of DAMGO induced an increase in phospho-Ser stimulation of EGFR, suggesting that EGFR is desensitized before its internalization, consistent with current concepts of the desensitization sequela (Kim et al., 2003). Ser phosphorylation of EGFR by U69,593 was also observed after a 10-min treatment (Fig. 7B).

**MEK Inhibition Blocks MOR-Induced EGFR Down-Regulation.** Down-regulation of EGFR may be blocked by inhibitors of various Ser/Thr kinases, including MAP kinases. The involvement of ERK in the mechanism of EGFR down-regulation by long-term DAMGO was studied in astrocytes overexpressing MOR. Time course studies of DAMGO treatment of a longer duration than determined previously revealed that EGFR levels decreased by 45% in 4 h (Fig. 8). These results extend the data obtained on EGFR down-regulation via endogenous MOR (Fig. 6, A and B). Moreover, the MEK inhibitor U0126 blocked EGFR down-regulation induced by long-term (2 h) DAMGO administration, supporting the notion that ERK activation is required for EGFR down-regulation by long-term MOR agonists.

## Discussion

The results obtained here suggest that the μ opioid agonist, DAMGO, acting through MOR, initially transactivates EGFR and subsequently implements the internalization and down-regulation of this RTK in a time-dependent manner. These findings may explain the ephemeral heterologous ERK activation by short-term DAMGO administration and the ultimate attenuation of EGF-stimulated ERK activation by long-term DAMGO administration in rat astrocytes. Accordingly, the κ opioid agonist U69,593 transactivated EGFR but did not down-regulate this RTK or inhibit EGF-stimulated ERK phosphorylation. The data are consonant with the sustained ERK phosphorylation induced by activation of KOR in rat astrocytes (Fig. 1B). Also consistent with these findings is the observation that long-term treatment with EGF diminishes cell surface binding to 10% within 30 min and reduces ERK phosphorylation to basal levels within 120 min.

Evidence for the transactivation of EGFR by DAMGO and U69,593 rests upon the attenuation of their stimulation of ERK phosphorylation by the selective EGFR Tyr kinase inhibitor tyrphostin AG1478 and the ability of these opioids to induce EGFR Tyr phosphorylation. The results suggest that MOR and KOR transactivation of EGFR represents the point

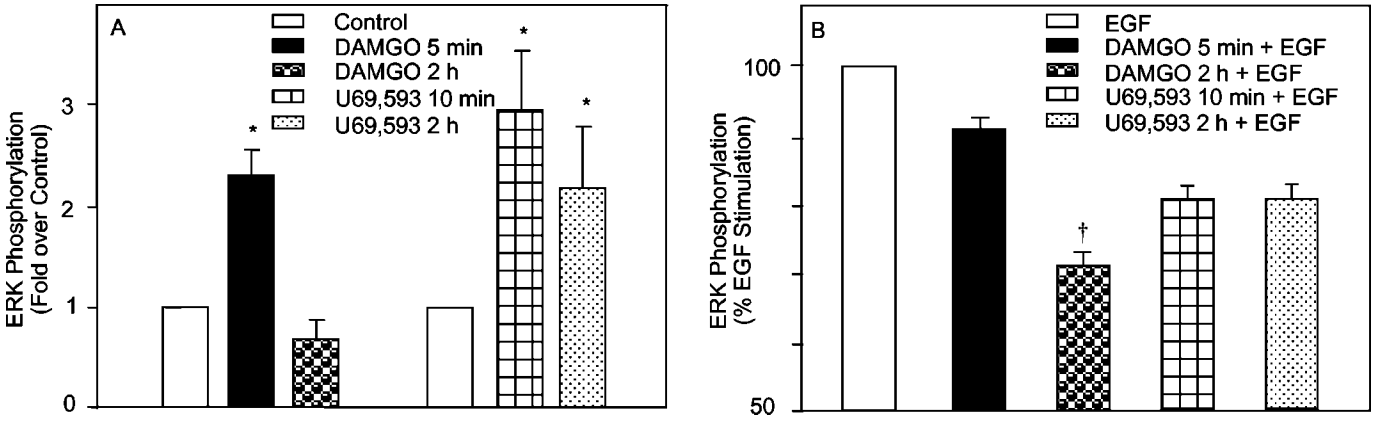


**Fig. 5.** Effects of short- and long-term μ and κ opioids on ERK phosphorylation in the presence and absence of EGF. Astrocytes were treated with DAMGO (0.1 μM, 5 min or 2 h) or U69,593 (0.1 μM, 10 min or 2 h) (A) or the same opioid treatment followed by EGF (0.1 μg/ml, 3 min) (B) and ERK phosphorylation was measured. Data in A are expressed as -fold over control and data in B are expressed as percentage of EGF stimulation of ERK. EGF stimulation of ERK was 13 ± 1.9-fold greater than basal levels. The 20% decreases in ERK activity observed when U69,593 (10 min and 2 h) were assayed in the presence of EGF in B were not statistically significant ($P = 0.12$ and 0.053, respectively). $n = 3$–9. *, significantly more than control; †, $P < 0.05$; significantly less than EGF.

of convergence of opioid and EGF signaling pathways in rat astrocytes. GPCR agonists may initiate EGFR transactivation by stimulating metalloproteinases that are involved in ectodomain shedding of endogenously expressed growth factor-like ligands. Accordingly, pretreatment of rat astrocytes with three metalloproteinase inhibitors, which act at different sites, significantly reduced MOR and KOR stimulation of ERK phosphorylation (Fig. 4).

A number of diverse mechanisms trigger the negative regulation of ERK in cells. Most of these mechanisms entail cross-talk between GPCR and RTK pathways. In earlier studies, it was recognized that secondary effectors, such as PKC, protein kinase A, and $Ca^{2+}$/calmodulin kinase, were capable of inhibiting ERK phosphorylation (Cochet et al., 1984; Countaway et al., 1992; Bosch et al., 1998). More recently, other signaling components, such as lipid phosphate phosphatases, have been found to negatively regulate ERK phosphorylation (Alderton et al., 2001).

If an RTK transactivation mechanism is involved in GPCR heterologous activation of ERK, then the RTK becomes a target for feedback regulation. The mechanisms of regulation of ERK by secondary effectors of GPCR pathways seem to operate as feedback loops acting on EGFR with differential efficacy. The phosphorylation of certain Ser/Thr residues of EGFR by secondary effectors reduces EGF binding and induce desensitization and, in some cases, down-regulation of

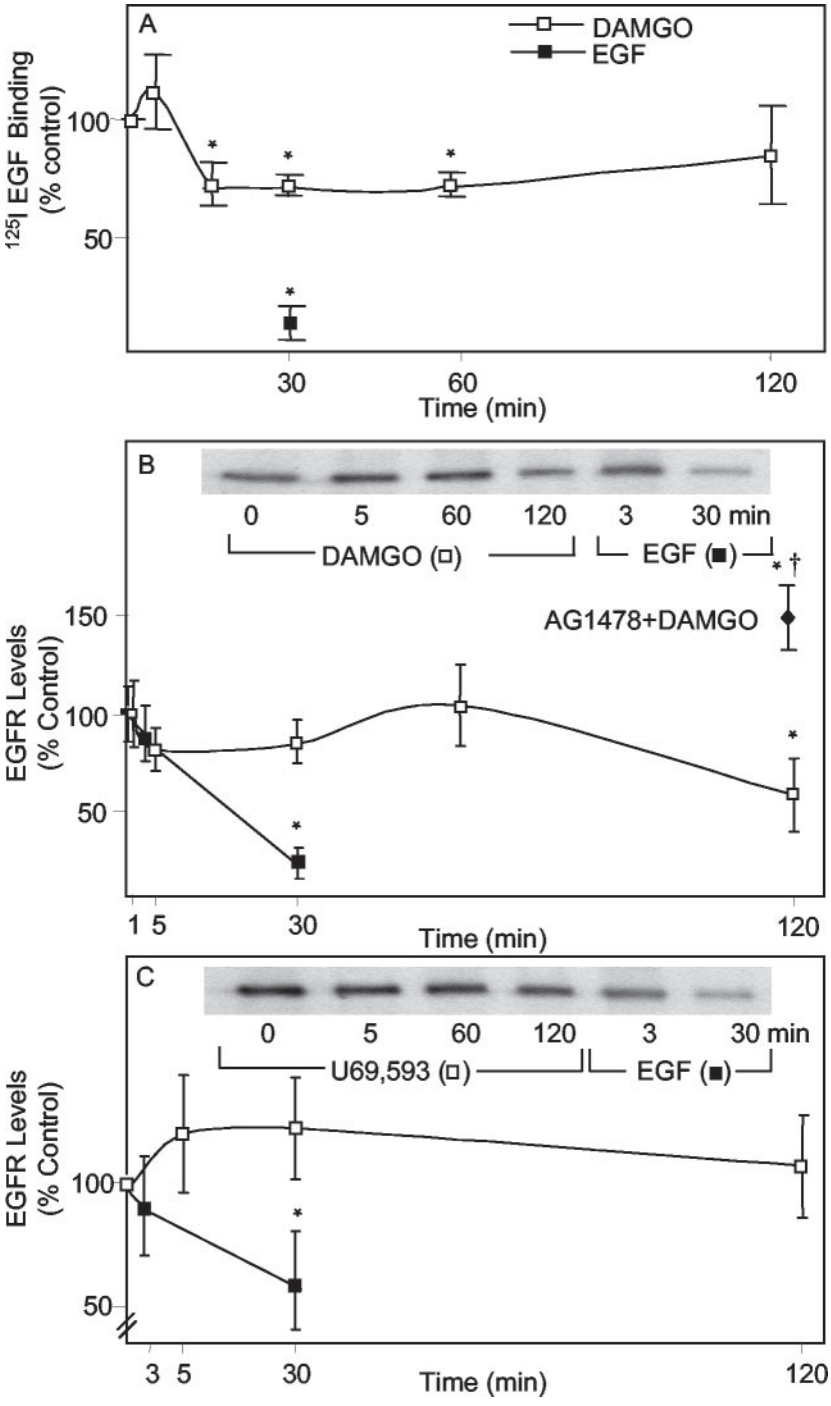


**Fig. 6.** Time course of MOR but not KOR induced EGFR internalization and down-regulation. A, DAMGO induced changes in cell surface EGFR binding. Astrocytes were treated with DAMGO (0.1 $\mu$M) or EGF (0.1 $\mu$g/ml) for different time points, and $^{125}$I-EGF binding to cell surface EGFR was measured. $n$ = 3–5. B and C, opioid effects on EGFR levels. Astrocytes were treated with DAMGO (0.1 $\mu$M) (B), U69,593 (0.1 $\mu$M) or EGF (0.1 $\mu$g/ml) (C) for the indicated time intervals. In some experiments, cells were pretreated with AG1478 (0.1 $\mu$M) for 20 min before exposure to DAMGO for 2 h. Levels of EGFR were determined by immunoblotting with an anti-EGFR antibody as described under *Materials and Methods*. Treatment of cells with AG1478 alone did not change EGFR levels; their content was 1 ± 0.09 compared with controls. $n$ = 3–12 experiments. *, $P < 0.05$; significantly less than control. †, $P < 0.05$; significantly more than DAMGO alone at 2 h.

the receptor. Cyclin-dependent kinase, such as *cdc2*, can phosphorylate EGFR on Ser1002 in vitro and in vivo (Kuppuswamy et al., 1993). ERK can also activate an EGFR-associated phosphatase activity that inhibits EGFR Tyr phosphorylation (Griswold-Prenner et al., 1993). Desensitization of EGFR is mediated by several Ser/Thr kinases, including PKC, protein kinase A, and ERK (Countaway et al., 1992; Kuppuswamy et al., 1993; Morrison et al., 1993, 1996). Ser 1046 of EGFR is the site for phosphorylation by $Ca^{2+}$/calmodulin kinase II; thus, another possibility for a feedback signaling pathway exists (Countaway et al., 1992). PKC inhibition of EGFR occurs by phosphorylation at Thr 654, which decreases its intrinsic Tyr kinase activity. The phosphorylation of this Thr residue diverts internalized EGFR from a degradative pathway to the recycling endosome (Bao et al., 2000). EGFR seems to be phosphorylated on Thr 669 by ERK (Northwood et al., 1991; Takishima et al., 1991).

Our working hypothesis is that long-term μ agonists may modulate the EGFR down-regulation via two steps. The first is to change the Ser/Thr phosphorylation pattern of EGFR that leads to the second process, which is to divert EGFR trafficking from early endosomes to lysosomes in astrocytes. The first part of this hypothesis is based on the evidence that, depending upon the Ser/Thr residues phosphorylated, sorting of internalized EGFR to lysosomes or recycling endosomes occurs (Bao et al., 2000). Therefore, it is important to determine the Ser/Thr phosphorylation pattern and the kinase(s) involved in this process. The 59% increase in EGFR levels by a 2-h treatment of cells with an AG1478/DAMGO combination (Fig. 6B) is consistent with the hypothesis that ERK is down-regulating EGFR via Ser phosphorylation. The ERK-induced EGFR Ser phosphorylation might be alleviated by AG1478, reversing its down-regulation and promoting its up-regulation.

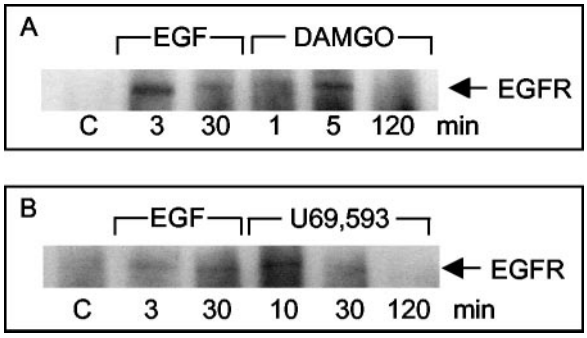


**Fig. 7.** Opioid induction of EGFR-Ser phosphorylation. Representative gels of opioid-induced EGFR-Ser phosphorylation. Astrocytes were treated with DAMGO (0.1 μM) in gel (A); U69,593 (0.1 μM) in gel (B), or EGF (0.1 μg/ml in both gels). In both gels, lane 1 (C) serves as a control. EGFR was immunoprecipitated and immunoblotting was performed with a phospho-Ser antibody. Similar gels were obtained in four experiments.

Currently there is no satisfactory mechanism or model to explain the PKC effects on EGFR internalization and down-regulation. It has been demonstrated that a MEK inhibitor, blocks PKC-mediated down-regulation of EGF-stimulated EGFR kinase activity but does not perturb PKC-mediated down-regulation of high-affinity EGF binding (Morrison et al., 1996). In addition, it was found that PKC inhibition blocks 5-HT induced down-regulation of EGFR in mesangial cells (Grewal et al., 2001). These findings are not consistent with the data of Bao et al., (2000). In preliminary studies, we discovered that DAMGO- and U69,593-induced ERK activation displays a differential sensitivity to PKC inhibitors in rat astrocytes (M. M. Belcheva, A. L. Clark, and P. D. Haas, unpublished observations). These results raise the possibility that MOR and KOR may activate ERK via two different isoforms of PKC: the MOR-associated PKC that engenders a Ser/Thr phosphorylation pattern that causes EGFR down-regulation, whereas the PKC isoform of the KOR pathway induces the phosphorylation of different Ser/Thr that does not induce down-regulation. Because PKC is necessary for opioid activation of ERK, direct experiments with PKC inhibitors are ruled out and different approaches to address this complex question are underway.

Here, we decided to focus on the role of ERK in MOR-induced EGFR down-regulation. ERK activation has been found to be necessary for MOR homologous desensitization (Polakiewicz et al., 1998) and reciprocal heterologous desensitization of EGFR by opioids seemed to have the same requirement (Fig. 8). Secondly, ERK can also alter the Ser/Thr pattern of EGFR phosphorylation as discussed above. There is also a growing body of evidence suggesting that MAP

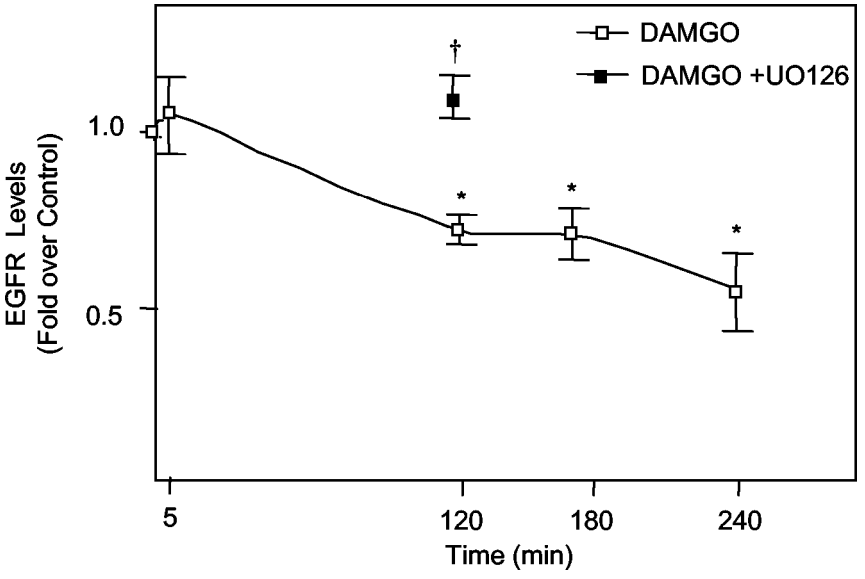


**Fig. 8.** DAMGO-induced down-regulation of EGFR in MOR-transfected rat astrocytes is abolished by MEK inhibitor U0126. Astrocytes transfected with MOR cDNA were treated with 0.1 μM DAMGO for the indicated time intervals. In some experiments, cells were pretreated with 10 μM U0126 for 1 h before treatment with DAMGO for 2 h. Levels of EGFR were determined by immunoblotting with an anti-EGFR antibody. U0126 treatment alone did not differ significantly from control values (1.1 ± 0.12). $n$ = 3–7. *, significantly less than controls; †, $P < 0.05$, significantly more than DAMGO, 2 h.

kinases are mediators of both positive and negative feedback loops in many cell lines (Bhalla et al., 2002). This advocates a role for MAP kinase in neural plasticity. It will be of interest to determine whether EGFR Ser phosphorylation and thereby heterologous desensitization also requires activation of the MAP kinase phosphorylation cascade. Although mechanistic studies of this type are facilitated by the in vitro approach adopted here, the use of cultured astrocytes has some limitations. Nevertheless, the information gained will expedite the in vivo investigations necessary to establish the generality of our results.

Collectively, the findings suggest that MOR plays a stimulatory (short-term actions) and an inhibitory (long-term actions) role in the heterologous regulation of EGFR and ERK activity in rat astrocytes. Thus, the results provide a mechanism for the attenuation of astrocyte proliferation by long-term opiates observed in vitro and in vivo. Moreover, this study on the mechanism of opioid regulation of ERK activation gains significance in light of the recent evidence of the role that astrocytes play during neurogenesis in adult brain.

## References


Alderton F, Darroch P, Sambi B, McKie A, Ahmed IS, Pyne N, and Pyne S (2001) G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1, 1a, and 2 in human embryonic kidney 293 cells. *J Biol Chem* **276:**13452–13460.

Bao J, Alroy I, Waterman H, Schejter ED, Brodie C, Gruenberg J, and Yarden Y (2000) Threonine phosphorylation diverts internalized epidermal growth factor receptors from a degradative pathway to the recycling endosome. *J Biol Chem* **275:**26178–26186.

Beitner-Johnson D, Guitart X, and Nestler EJ (1993) Glial fibrillary acidic protein and the mesolimbic dopamine system: regulation by chronic morphine and Lewis-Fischer strain differences in the rat ventral tegmental area. *J Neurochem* **61:** 1766–1773.

Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, Levy R, Young EC, Barg J, and Coscia CJ (1998) Opioid modulation of extracellular signal regulated protein kinase activity is Ras dependent and involves G$\beta\gamma$ subunits. *J Neurochem* **70:**635–645.

Belcheva MM, Wong YH, and Coscia CJ (2000) Evidence for transduction of mu but not kappa opioid modulation of extracellular signal-regulated kinase activity by G(z) and G(12) proteins. *Cell Signal* **12:**481–489.

Belcheva MM, Haas PD, Tan Y, Heaton VM, and Coscia CJ (2002) The fibroblast growth factor receptor is at the site of convergence between mu-opioid receptor and growth factor signaling pathways in rat C6 glioma cells. *J Pharmacol Exp Ther* **303:**909–918.

Belcheva MM, Szucs M, Wang DX, Sadee W, and Coscia CJ (2001) Mu-opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. *J Biol Chem* **276:**33847–33853.

Berhow MT, Hiroi N, and Nestler EJ (1996) Regulation of Erk (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* **16:**4707–4715.

Bhalla US, Ram PT, and Iyengar R (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science (Wash DC)* **297:**1018–1023.

Bohn LM, Belcheva MM, and Coscia CJ (2000a) Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: Evidence for the involvement of protein kinase C and the mitogen-activated protein kinase signaling cascade. *J Neurochem* **74:**564–573.

Bohn LM, Belcheva MM, and Coscia CJ (2000b) Mu-opioid agonist inhibition of kappa-opioid receptor-stimulated extracellular signal-regulated kinase phosphorylation is dynamin-dependent in C6 glioma cells. *J Neurochem* **74:**574–581.

Bosch M, Gil J, Bachs O, and Agell N (1998) Calmodulin inhibitor W13 induces sustained activation of ERK2 and expression of P21(CIP1). *J Biol Chem* **273:** 22145–22150.

Cochet C, Gill GN, Meisenhelder J, Cooper JA, and Hunter T (1984) C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* **259:**2553–2558.

Countaway JL, Nairn AC, and Davis RJ (1992) Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. *J Biol Chem* **267:**1129–1140.

Daub H, Weiss FU, Wallasch C, and Ullrich A (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature (Lond)* **379:** 557–560.

Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, and Luttrell LM (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade—role of focal adhesions and receptor tyrosine kinases. *J Biol Chem* **274:**13978–13984.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, and Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97:**703–716.

Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, and Alvarez-Buylla A (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron* **36:**1021–1034.

Eguchi S, Matsumoto T, Motley ED, Utsunomiya H, and Inagami T (1996) Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells - Possible requirement of $G_q$-mediated P21(Ras) activation coupled to a $Ca^{2+}$/calmodulin-sensitive tyrosine kinase. *J Biol Chem* **271:**14169–14175.

Eisch AJ, Barrot M, Schad CA, Self DW, and Nestler EJ (2000) Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci USA* **97:**7579–7584.

Eriksson PS, Hansson E, and Ronnback L (1991) Mu and delta opiate receptors in neuronal and astroglial primary cultures from various regions of the brain–coupling with adenylate cyclase, localisation on the same neurones and association with dopamine (D1) receptor adenylate cyclase. *Neuropharmacology* **30:**1233–1239.

Finklestein SP, Apostolides PJ, Caday CG, Prosser J, Philips MF, and Klagsbrun M (1988) Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of focal brain wounds. *Brain Res* **460:**253–259.

Grewal JS, Luttrell LM, and Raymond JR (2001) G protein-coupled receptors desensitize and down-regulate epidermal growth factor receptors in renal mesangial cells. *J Biol Chem* **276:**27335–27344.

Griswold-Prenner I, Carlin CR, and Rosner MR (1993) Mitogen-activated protein kinase regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase. *J Biol Chem* **268:**13050–13054.

Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G, and Gudermann T (1998) Ligand-independent activation of platelet-derived growth-factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. *Proc Natl Acad Sci USA* **95:**8985–8990.

Kim J, Ahn S, Guo R, and Daaka Y (2003) Regulation of epidermal growth factor receptor internalization by G protein-coupled receptors. *Biochemistry* **42:**288–2894.

Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Derynck R, and Werb Z (1998) Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. *J Neurosci Res* **53:**697–717.

Kuppuswamy D, Dalton M, and Pike LJ (1993) Serine 1002 is a site of in vivo and in vitro phosphorylation of the epidermal growth factor receptor. *J Biol Chem* **268:** 19134–19142.

Learish RD, Bruss MD, and Haak-Frendscho M (2000) Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. *Dev Brain Res* **122:**97–109.

Linseman DA, Benjamin CW, and Jones DA (1995) Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells. *J Biol Chem* **270:**12563–12568.

Morrison P, Saltiel AR, and Rosner MR (1996) Role of mitogen-activated protein kinase kinase in regulation of the epidermal growth factor receptor by protein kinase C. *J Biol Chem* **271:**12891–12896.

Morrison P, Takishima K, and Rosner MR (1993) Role of threonine residues in regulation of the epidermal growth factor receptor by protein kinase-C and mitogen-activated protein kinase. *J Biol Chem* **268:**15536–15543.

Nieto-Sampedro M, Gomez-Pinilla F, Knauer DJ, and Broderick JT (1988) Epidermal growth factor receptor immunoreactivity in rat brain astrocytes. Response to injury. *Neurosci Lett* **91:**276–282.

Northwood IC, Gonzalez FA, Wartmann M, Raden DL, and Davis RJ (1991) Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J Biol Chem* **266:**15266–15276.

Opanashuk LA and Hauser KF (1998) Opposing actions of the EGF family and opioids-heparin binding epidermal growth factor (HB-EGF) protects mouse cerebellar neuroblasts against the antiproliferative effect of morphine. *Brain Res* **804:**87–94.

Persson AI, Thorlin T, Bull C, Zarnegar P, Ekman R, Terenius L, and Eriksson PS (2003) Mu- and delta-opioid receptor antagonists decrease proliferation and increase neurogenesis in cultures of rat adult hippocampal progenitors. *Eur J Neurosci* **17:**1159–1172.

Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, and Lefkowitz RJ (2001) Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* **276:** 23155–23160.

Polakiewicz RD, Schieferl SM, Dorner LF, Kansra V, and Comb MJ (1998) A mitogen-activated protein kinase pathway is required for mu-opioid receptor desensitization. *J Biol Chem* **273:**12402–12406.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, and Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature (Lond)* **402:**884–888.

Radany EH, Brenner M, Besnard F, Bigornia V, Bishop JM, and Deschepper CF (1992) Directed establishment of rat brain cell lines with the phenotypic characteristics of type 1 astrocytes. *Proc Natl Acad Sci USA* **89:**6467–6471.

Roudabush FL, Pierce KL, Maudsley S, Khan KD, and Luttrell LM (2000) Transactivation of the EGF receptor mediates IGF-1-stimulated Shc phosphorylation and ERK1/2 activation in COS-7 cells. *J Biol Chem* **275:**22583–22589.

Ruzicka BB, Fox CA, Thompson RC, Meng F, Watson SJ, and Akil H (1995) Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. *Brain Res Mol Brain Res* **34:**209–220.

Song H, Stevens CF, and Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. *Nature (Lond)* **417:**39–44.

Sorkin A, Mazzotti M, Sorkina T, Scotto L, and Beguinot L (1996) Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. *J Biol Chem* **271:**3377–3384.
Stiene-Martin A and Hauser KF (1991) Glial growth is regulated by agonists selective for multiple opioid receptor types in vitro. *J Neurosci Res* **29:**538–548.
Stiene-Martin A, Knapp PE, Martin K, Gurwell JA, Ryan S, Thornton SR, Smith FL, and Hauser KF (2001) Opioid system diversity in developing neurons, astroglia and oligodendroglia in the subventricular zone and striatum: Impact on gliogenesis *in vivo*. *Glia* **36:**78–88.
Takishima K, Griswold-Prenner I, Ingebritsen T, and Rosner MR (1991) Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3–L1 cells is an EGF-stimulated "MAP" kinase. *Proc Natl Acad Sci USA* **882:**520–524.
Yan Y, Shirakabe K, and Werb Z (2002) The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. *J Cell Biol* **158:**221–226.

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