

# Epidermal Growth Factor Treatment Switches $\delta$ -Opioid Receptor-Stimulated Extracellular Signal-Regulated Kinases 1 and 2 Signaling from an Epidermal Growth Factor to an Insulin-Like Growth Factor-1 Receptor-Dependent Mechanism

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## ABSTRACT

$\delta$ -Opioid receptor (DOR)-induced activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) is mediated by the transactivation of epidermal growth factor (EGF) receptors. Here we demonstrate that in stably DOR-expressing human embryonic kidney (HEK) 293 (HEK/DOR) cells, down-regulation of EGF receptors by long-term EGF (0.1  $\mu$ g for 18 h) treatment, but not by small interfering RNA, results in functional desensitization of EGF (10 ng/ml)-stimulated ERK1/2 signaling. In EGF receptor-desensitized (HEK/DOR<sup>-EGFR</sup>) cells, however, [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin (1  $\mu$ M) and etorphine (0.1  $\mu$ M) retained their ability to stimulate ERK1/2 activation. The newly acquired signal transduction mechanism is insensitive to the EGF receptor blockers 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) and *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butyramide (CL-387,785), does not involve DOR internalization and activation of the focal adhesion kinase pp125FAK, but requires matrix metalloproteinase-dependent release of soluble growth factors. A supernatant transfer assay in which conditioned growth media

of opioid-treated HEK/DOR and HEK/DOR<sup>-EGFR</sup> “donor” cells are used to stimulate ERK1/2 activity in DOR-lacking HEK293 wild type and HEK293<sup>-EGFR</sup> “acceptor” cells revealed that long-term EGF treatment produces a switch in the receptor tyrosine kinase (RTK) system transactivated by opioids. Using microfluidic electrophoresis, chemical inhibitors, phosphorylation-specific antibodies, and EGF receptor-deficient Chinese hamster ovary-K1 cells, we identified the release of an insulin-like growth factor-1 (IGF-1)-like peptide and activation of IGF-1 receptors in HEK/DOR<sup>-EGFR</sup> cells after DOR activation. A similar switch from a neurotrophic tyrosine kinase receptor type 1 to an IGF-1 receptor-dependent ERK1/2 signaling was observed for chronically nerve growth factor-treated neuroblastoma  $\times$  glioma (NG108-15) cells. These results indicate that transactivation of the dominant RTK system in a given cellular setting may represent a general feature of opioids to maintain mitogenic signaling.

## Introduction

In many cell types and tissues, G protein-coupled receptors stimulate mitogenic signaling via “transactivation” of receptor tyrosine kinases (RTKs), including the epidermal growth factor (EGF) receptor (Luttrell, 2005). The transactivation

process involves activation of membrane-bound matrix metalloproteinases (MMPs), which regulate the proteolytic release of EGF-like growth factors from the cell surface (Prenzel et al., 1999). Whereas the precise mechanisms by which GPCRs stimulate MMP activity largely remain unclear (Ohtsu et al., 2006), the molecular events of EGF receptor-mediated activation of mitogenic signaling are well defined. Once transactivated by their cognate ligands, the EGF receptor becomes autophosphorylated at multiple cytoplasmic ty-

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**ABBREVIATIONS:** RTK, receptor tyrosine kinase; Con A, concanavalin A; DADLE, [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin; CHO, Chinese hamster ovary; DOR,  $\delta$ -opioid receptor; EGCG, epigallocatechin-3-gallate; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinases 1/2; FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; HB-EGF, heparin binding epidermal growth factor; HEK, human embryonic kidney; IGF-1, insulin-like growth factor type 1; MMP, matrix metalloproteinase; NGF, nerve growth factor; wt, wild type; TrkA, neurotrophic tyrosine kinase receptor type 1; CL-387,785, *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butyramide; HA, hemagglutinin; FCS, fetal calf serum; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; SOCS-3, suppressor of cytokine signaling 3; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; AG538,  $\alpha$ -cyano-(3-methoxy,4-hydroxy,5-iodo)cinnamoyl-(3',4'-dihydroxyphenyl)ketone; AG1295, 6,7-dimethyl-2-phenylquinoxaline; AG1024, 3-bromo-5-*t*-butyl-4-hydroxy-benzylidenemalo-nitrile; AG879,  $\alpha$ -cyano-(3,5-di-*t*-butyl-4-hydroxy)thiocinnamide; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; GM6001, *N*-[(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide.

rosine residues (e.g., Tyr992, Tyr1045, Tyr1068, Tyr1148, and Tyr1173), providing docking sites for Src-homology domain 2-containing proteins such as SHC and Gab1 (Downward et al., 1984). After phosphorylation, the adapter proteins recruit the Ras guanine-nucleotide exchange factor complex Grb2 · Sos1 to the activated EGF receptor, which then activates the conserved Raf/mitogen-activated protein kinase kinase/ERK1/2 signaling module (Rozakis-Adcock et al., 1993).

Besides their analgesic action,  $\delta$ -opioid receptor (DOR) agonists also display proliferative and antiapoptotic properties (Chen et al., 2008). The growth promoting effects of opioids are mediated through transactivation of EGF receptor-associated mitogenic signaling pathways (Tegeder and Geisslinger, 2004). In HEK293 cells, transactivation of EGF receptors involves ectodomain shedding of EGF-like ligands by activated MMP-2 and MMP-9 isoforms (Schulz et al., 2004; Eisinger and Ammer, 2008a). Although transactivation of the EGF receptor system is believed to be a general concept by which opioids mediate ERK1/2 signaling, mitogenic signaling was also observed in DOR-transfected C6 glioma cells lacking endogenous EGF receptors (Kramer et al., 2002). Because we have described that in neuroblastoma  $\times$  glioma (NG108-15) hybrid cells DOR-induced ERK1/2 signaling involves transactivation of neurotrophic tyrosine kinase receptor type 1 (TrkA) receptors (Eisinger and Ammer, 2008b), these observations raise the question of whether other RTK systems may generally substitute for EGF receptors in mediating opioid-induced ERK1/2 signaling or whether additional intracellular pathways exist.

Here we examine the consequences of functional inactivation of the dominant EGF receptor system on the signal transduction mechanism mediating DOR-stimulated ERK1/2 activation in HEK293 cells. Our results demonstrate that EGF receptor down-regulation and desensitization by long-term EGF treatment does not abrogate but rather establishes a novel pathway by which opioids maintain mitogenic signaling by an EGF receptor-independent manner. The newly acquired signal transduction pathway involves the release of an insulin-like growth factor-1 (IGF-1)-like peptide and transactivation of IGF-1 receptors that directly stimulate the Ras/Raf/mitogen-activated protein kinase kinase/ERK1/2 signaling cascade. Because long-term nerve growth factor (NGF) treatment of NG108-15 hybrid cells results in a similar switch from a neurotrophic tyrosine kinase receptor type 1 (TrkA) to IGF-1 receptor-mediated signaling mechanism, these results demonstrate that the IGF-1 receptor system may mediate opioid-induced ERK1/2 signaling after functional inactivation of the dominant growth factor system. They also indicate that the recruitment of alternative RTK systems ensures the continued control of mitogenic pathways by opioids in the absence of functionally active EGF receptors.

## Materials and Methods

**Materials.** [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>]enkephalin (DADLE) and recombinant human EGF, heparin binding EGF (HB-EGF), and IGF-1 were purchased from Bachem (Bubendorf, Switzerland). Etorphine was obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD). The small-molecule inhibitors  $\alpha$ -cyano-(3,5-di-*t*-butyl-4-hydroxy)thiocinnamide (AG879), 3-bromo-5-*t*-butyl-4-

hydroxy-benzylidenemalonitrile (AG1024), 6,7-dimethyl-2-phenylquinoline (AG1295), 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), and *N*-[4-[(3-bromophenyl)amino]-6-quinazolyl]-2-butyramide (CL-387,785) were from Merck Biosciences (Schwalbach, Germany),  $\alpha$ -cyano-(3-methoxy,4-hydroxy,5-iodo)cinnamoyl-(3',4'-dihydroxyphenyl)ketone (I-OMe-Tyr-phostin; AG538), concanavalin A (Con A), and epigallocatechin-3-gallate (EGCG) were from Sigma-Aldrich (Deisenhofen, Germany). The following antibodies were from Cell Signaling Technology (Danvers, MA): anti-ERK1/2, anti-phospho-ERK1/2, anti-IGF-1 receptor  $\beta$ -subunit, anti-phospho(Tyr1135/1136)IGF-1 receptor  $\beta$ -subunit, anti-phospho-pp125FAK (all rabbit polyclonal antibodies), and anti-EGF receptor (mouse monoclonal antibody, clone 1F4). Rabbit polyclonal anti-hemagglutinin (HA) epitope, anti-c-Myc tag, and anti-G protein  $\beta_{1/2}$ -subunit IgGs were from Gramsch Laboratories (Schwabhausen, Germany), anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies from Dianova GmbH (Hamburg, Germany). Control and target-specific small interfering RNAs (siRNAs) for the EGF and IGF-1 receptor were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid pcDNA3 containing a c-Myc-tagged variant of SOCS-3 cDNA was a kind gift from Dr. Akihiko Yoshimura (Division of Molecular and Cellular Immunology, Kyushu University, Kyushu, Japan). The plasmid containing the DOR fused to enhanced green fluorescent protein (pEGFP-DOR; Clontech, Mountain View, CA) was described earlier (Eisinger and Schulz, 2004). Fetal calf serum (FCS) and cell culture medium and supplements were from Invitrogen (Carlsbad, CA); standard laboratory chemicals were from Sigma-Aldrich.

**Cell Culture, Modification of the EGF Receptor System, and Drug Treatment.** Wild-type (HEK293 *wt*), stably DOR-expressing HEK293 cells (HEK/DOR; 1.4 pmol/mg membrane protein), and Chinese hamster ovary (CHO-K1) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C (Schulz et al., 2004). NG108-15 hybrid cells were cultured as described previously (Eisinger and Ammer, 2008b). For some experiments, HEK/DOR and CHO-K1 cells were transiently transfected with 5  $\mu$ g/100-mm dish of plasmid pcDNA3 (Invitrogen) containing c-Myc-SOS-3 or mouse DOR cDNA using Metafectene transfection reagent (Biontex, Martinsried, Germany) as described earlier (Eisinger and Ammer, 2008a). Transfection of HEK/DOR and CHO-K1 cells with siRNAs was performed by using MetafecteneSI transfection reagent (Biontex).

The day before experimentation, HEK cells were seeded onto 12-well tissue culture plates and allowed to grow overnight either in the absence or presence of EGF (0.1  $\mu$ g/ml; 18 h) to desensitize the predominant RTK system in this cell line. The resulting EGF-insensitive cells were designated HEK293<sup>-EGFR</sup> and HEK/DOR<sup>-EGFR</sup> throughout the study. NG108-15 cells were continuously treated with NGF (100  $\mu$ g/ml; 18 h) to desensitize TrkA receptors (NG108-15<sup>-Trk</sup> cells).

For the determination of ERK1/2 activity, cells were washed and equilibrated for 2 h in DMEM containing 10 mM HEPES, pH 7.4, and 0.1% FCS, to reduce basal mitogenic signaling. During serum withdrawal, the following drug treatments were performed: agonist-stimulated DOR internalization was blocked by the addition of Con A (250  $\mu$ g/ml, 30 min) and hypertonic sucrose (0.4 M, 2 h); transactivation of EGF, PDGF, TrkA, and IGF-1 receptors was inhibited by 30-min exposure to AG1478 (5  $\mu$ M) or CL-387,785 (1  $\mu$ M), AG1295 (50  $\mu$ M), AG879 (100  $\mu$ M), and AG1024 (10  $\mu$ M) or AG538 (100  $\mu$ M), respectively; matrix metalloproteinases were inactivated by the addition of the MMP blocker *N*-[(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001; 2  $\mu$ M, 60 min) or the green tea phenol epigallocatechin gallate (EGCG; 50  $\mu$ M, 2 h). Controls were kept in the presence of DMEM/0.1% FCS alone. At the end of the equilibration period, receptor-mediated stimulation of ERK1/2 activity was determined using maximum effective concentrations of the mixed  $\delta/\mu$ -peptide agonist DADLE (1  $\mu$ M) and the opioid alkaloid etorphine (0.1  $\mu$ M) and half-maximum effective con-

centrations of the growth factors EGF (10 ng/ml), IGF-1 (100 ng/ml), and NGF (100 ng/ml) for 5 min at 37°C. Reactions were stopped by aspiration of the medium and solubilization of the cells with 0.5 ml of Laemmli sample buffer [62.5 mM Tris-HCl, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% (w/v) phenol red, pH 6.8].

**Confocal Analysis of DOR Internalization.** To investigate the effect of long-term EGF treatment on inhibition of DOR internalization by Con A, HEK293 cells grown on coverslips were transiently transfected with EGFP-DOR. Cells were pretreated with EGF (0.1  $\mu$ g/ml) for 18 h to desensitize the EGF receptor. Cells were then exposed to Con A (250  $\mu$ g/ml) for 30 min before DOR internalization was initiated by the addition of opioids. At the end of the incubation period, cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with paraformaldehyde (4% in PBS buffer), and coverslips were mounted on object slides with Fluorescent Mounting Medium (Dako Deutschland GmbH, Hamburg, Germany). Confocal images of cell groups were obtained using a Zeiss LSM 510 microscope (Carl Zeiss GmbH, Jena, Germany). The images shown were acquired using a 63  $\times$  1.4 oil immersion objective.

**Cell Supernatant Assay.** To more closely define the role of RTK systems in opioid-stimulated ERK1/2 signaling, a novel two-step assay system was developed. This approach is based on the regulated release of growth factors by short-term treatment of HEK/DOR with opioids. The growth factor containing medium of these "donor" cells was subsequently transferred to DOR-deficient HEK293 *wt* "acceptor" cells to induce the stimulation of ERK1/2 activity. For this, HEK/DOR and HEK/DOR<sup>-EGFR</sup> "donor" cells grown on 12-well plates were washed three times with PBS, pH 7.4, and activated for 5 min with 1  $\mu$ M DADLE in 2 ml of DMEM/0.1% FCS, pH 7.4, at 37°C. Cells were chilled on ice; the "conditioned media" were collected, cleared by centrifugation (1000g, 10 min), and then added to thoroughly washed HEK293 *wt*, HEK293<sup>-EGFR</sup>, or CHO-K1 "acceptor" cells. Finally, acceptor cells were incubated for 5 min at 37°C to determine ERK1/2 phosphorylation, which was then detected by Western blot. In some experiments, acceptor cells were pretreated for 30 min with the following RTK blockers before stimulation with supernatants: AG1024 (10  $\mu$ M), AG879 (100  $\mu$ M), AG538 (100  $\mu$ M), AG1295 (50  $\mu$ M), and AG1478 (1  $\mu$ M).

**Western Blotting.** Whole-cell lysates in Laemmli sample buffer were cleared by centrifugation (1000g; 10 min), heated for 5 min to 95°C, and then resolved by electrophoresis over 10% (m/v) SDS-polyacrylamide gels. Proteins were subsequently blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA) and incubated with a rabbit polyclonal phosphospecific anti-ERK1/2 antibody to evaluate ERK1/2 activation as described previously (Eisinger and Schulz, 2004). The same samples were also probed with an overall reactive anti-ERK1/2 antibody to verify equal protein loading. Transient expression of c-Myc-SOCS-3 was confirmed using an anti-c-Myc-tag antibody. Relative changes in  $\delta$ -opioid receptor levels were analyzed in membranes from HEK293 cells stably expressing a HA-tagged variant of the rat DOR (HEK/HA-DOR; 0.8 pmol/mg membrane protein) using an anti-HA tag antibody. EGF and IGF-1 receptor abundances were determined either in membranes or whole-cell lysates from HEK/DOR cells using anti-EGF receptor and  $\beta$ -subunit-specific anti-IGF-1 receptor antibodies, respectively. The phosphorylation state of pp125FAK and the IGF-1 receptor was determined in lysates of HEK/DOR cells using phosphospecific antibodies. Immunocomplexes were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies using the enhanced chemiluminescence method (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Intensity of immunoreactive bands of total and phosphorylated ERK1/2 were quantitated by video densitometry using the Herolab EASY-5 system (Herolab GmbH, Wiesloch, Germany), and expressed as pERK/ERK ratio.

**Identification of Growth Factors.** Proteins from the incubation media of control and opioid-treated HEK/DOR and HEK/DOR<sup>-EGFR</sup> cells were precipitated with trichloroacetic acid (10% final concentration) for 30 min on ice. After centrifugation (15 min; 20,000g), the

proteins were washed with ice-cold acetone and solubilized in 200  $\mu$ l of Tris-HCl buffer (50 mM), pH 7.4. Electrophoresis samples were prepared according to the Protein 80 Kit manual instructions, loaded on lab-on-chip devices together with recombinant HB-EGF (2 ng/lane) and IGF-1 (6 ng/lane) as the standards, and analyzed by the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

## Results

In HEK/DOR cells, DOR-stimulated ERK1/2 signaling is mediated by transactivation of EGF receptors (Schulz et al., 2004). This is verified in the present study by preincubation of the cells with the reversible and irreversible EGF receptor inhibitors AG1478 (Levitzki and Gazit, 1995) and CL-387,785 (Discafani et al., 1999), respectively, which completely block DADLE (1  $\mu$ M)-, etorphine (0.1  $\mu$ M)-, and EGF (10 ng/ml)-induced Thr202/Tyr204 phosphorylation of ERK1/2. In addition, these results also indicate that in HEK/DOR cells, maximum effective concentrations of the stable opioid peptide agonist DADLE and the cell membrane-permeable alkaloid etorphine both stimulate ERK1/2 activity to the same extent by using a common signal transduction mechanism (Fig. 1A).

**Desensitization of EGF Receptors Does Not Affect Opioid-Induced ERK1/2 Signaling.** To investigate whether inactivation of the dominant EGF receptor system might possibly abrogate or redirect mitogenic opioid signaling to an alternative intracellular pathway, HEK/DOR cells were continuously exposed to EGF (0.1  $\mu$ g/ml; 18 h). This treatment regimen results in both down-regulation (Fig. 1B) and functional desensitization of EGF receptors as demonstrated by the failure of EGF (10 ng/ml; 5 min) to acutely stimulate ERK1/2 signaling.

The most critical finding of the present study is that functional desensitization of EGF receptors fails to block DOR-mediated activation of ERK1/2 signaling. Instead, long-term EGF treatment even enhances DADLE and etorphine-induced ERK1/2 activation by approximately  $55 \pm 14$  and  $49 \pm 17\%$  ( $n = 3$ ), a regulatory mechanism most likely mediated by up-regulation of DOR abundance after long-term EGF treatment (Fig. 1B). Because of the absence of functionally active EGF receptors in HEK/DOR<sup>-EGFR</sup> cells, DOR-mediated stimulation of ERK1/2 signaling must use an alternative mechanism. Indeed, pretreatment of the cells with the EGF receptor blockers AG1478 and CL-387,785 does not interfere with opioid-induced ERK1/2 phosphorylation (Fig. 1A). These results indicate that long-term EGF treatment seems to redirect DOR-stimulated ERK1/2 signaling to an EGF receptor-independent mechanism.

Down-regulation of EGF receptors was also examined after siRNA-mediated gene silencing. Although transfection of HEK/DOR cells with EGF receptor-specific siRNAs largely reduced EGF receptor immunoreactivity in whole-cell preparations, exposure of the cells to EGF (10 ng/ml), DADLE (1  $\mu$ M), and etorphine (100 nM) still resulted in strong degrees of ERK1/2 activation. In addition, DOR-mediated ERK1/2 signaling was abolished by pretreatment of the cells with the EGF receptor inhibitors AG1478 (5  $\mu$ M) and CL-387,785 (1  $\mu$ M) (Fig. 1C). These results indicate that down-regulation of EGF receptors by siRNA-mediated gene silencing seems insufficient to fully desensitize EGF receptor-associated signal transduction and to establish EGF receptor-independent mitogenic signaling. Thus, the present study was performed using continuously EGF (0.1  $\mu$ g/ml; 18 h)-treated HEK/DOR (HEK/DOR<sup>-EGFR</sup>) cells.



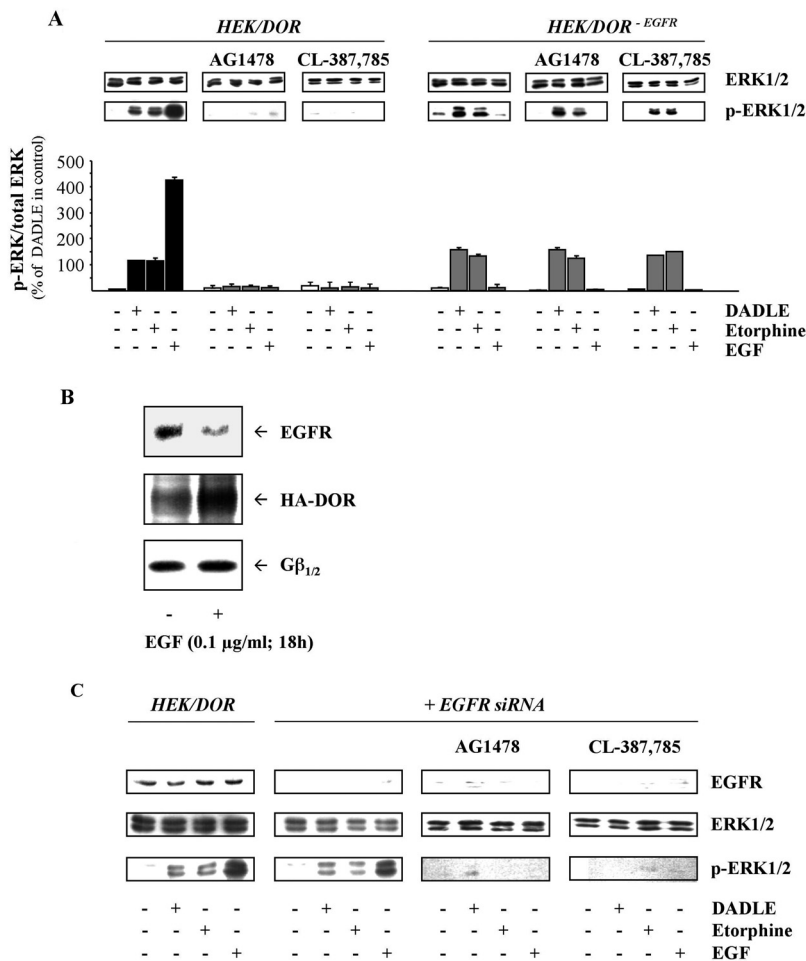
**DOR-Mediated ERK1/2 Signaling in HEK/DOR<sup>-EGFR</sup> Cells Involves MMP Activity.** To characterize the newly acquired DOR-associated signal transduction pathway in HEK/DOR<sup>-EGFR</sup> cells, we first discriminated whether an intracellular or an RTK-dependent paracrine mechanism is established. In HEK293 *wt* cells, agonist-induced receptor endocytosis represents a well defined mechanism by which sequestering GPCRs may bring about ERK1/2 activation (Pierce et al., 2000). However, pretreatment of HEK/DOR<sup>-EGFR</sup> cells with Con A, which blocks DOR endocytosis by DADLE and etorphine (Fig. 2A), had no effect on opioid-stimulated ERK1/2 activation (Fig. 2B). Similar results were obtained when receptor endocytosis was blocked by hypertonic sucrose (data not shown). As presented in Fig. 2C, Con A and hypertonic sucrose also failed to attenuate opioid-induced mitogenic signaling in DOR-transfected CHO-K1 cells lacking endogenous EGF receptors (Della Rocca et al., 1999). Together, these results indicate that receptor endocytosis does not contribute to DOR-mediated ERK1/2 signaling in HEK/DOR<sup>-EGFR</sup> cells.

We next examined whether desensitization of EGF receptors might possibly redirect DOR-stimulated ERK1/2 activation to an intracellular mechanism using the focal adhesion kinase pp125FAK (Della Rocca et al., 1999). HEK/DOR cells are characterized by only low levels of basal tyrosine-576/577 pp125FAK phosphorylation, which are not subject to regulation by opioids. Overexpression of a c-Myc-tagged construct of the pp125FAK inhibitor SOCS-3 (Eisinger and Ammer, 2008a) and subsequent exposure of the cells to EGF (0.1  $\mu$ g;

18 h) completely abolished phosphorylation and, thus, activation of pp125FAK. In contrast, c-Myc-SOCS-3 had no effect on overall abundance of ERK1/2 and on DOR-mediated ERK1/2 stimulation in HEK/DOR<sup>-EGFR</sup> cells (Fig. 2B). These results indicate that in HEK/DOR<sup>-EGFR</sup> cells DOR-stimulated ERK1/2 signaling is independent of focal adhesion tyrosine kinase activity.

Finally, we evaluated whether long-term EGF treatment would possibly switch opioid-induced ERK1/2 stimulation to transactivation of an alternative RTK system. Because GPCR-mediated transactivation of RTK systems requires the proteolytic cleavage of growth factors from the plasma membrane, the role of MMPs was examined in DOR-mediated ERK1/2 signaling. As shown in Fig. 2B, preincubation of HEK/DOR<sup>-EGFR</sup> cells with the broad spectrum MMP inhibitor EGCG (Demeule et al., 2000) almost completely blocked subsequent stimulation of ERK1/2 activity by DADLE and etorphine. Similar results were obtained when HEK/DOR<sup>-EGFR</sup> cells were pretreated with the nonselective MMP blocker GM6001 (Santiskulvong and Rozengurt, 2003). These findings suggest that in HEK/DOR<sup>-EGFR</sup> cells MMP-dependent transactivation of an alternative RTK system might play a critical role in the signal transduction pathway mediating opioid-stimulated ERK1/2 signaling.

**Long-Term EGF Treatment Recruits an Alternative RTK System Transactivated by Opioids.** To determine whether a paracrine mechanism might account for opioid-stimulated ERK1/2 signaling in HEK/DOR<sup>-EGFR</sup> cells, a two-

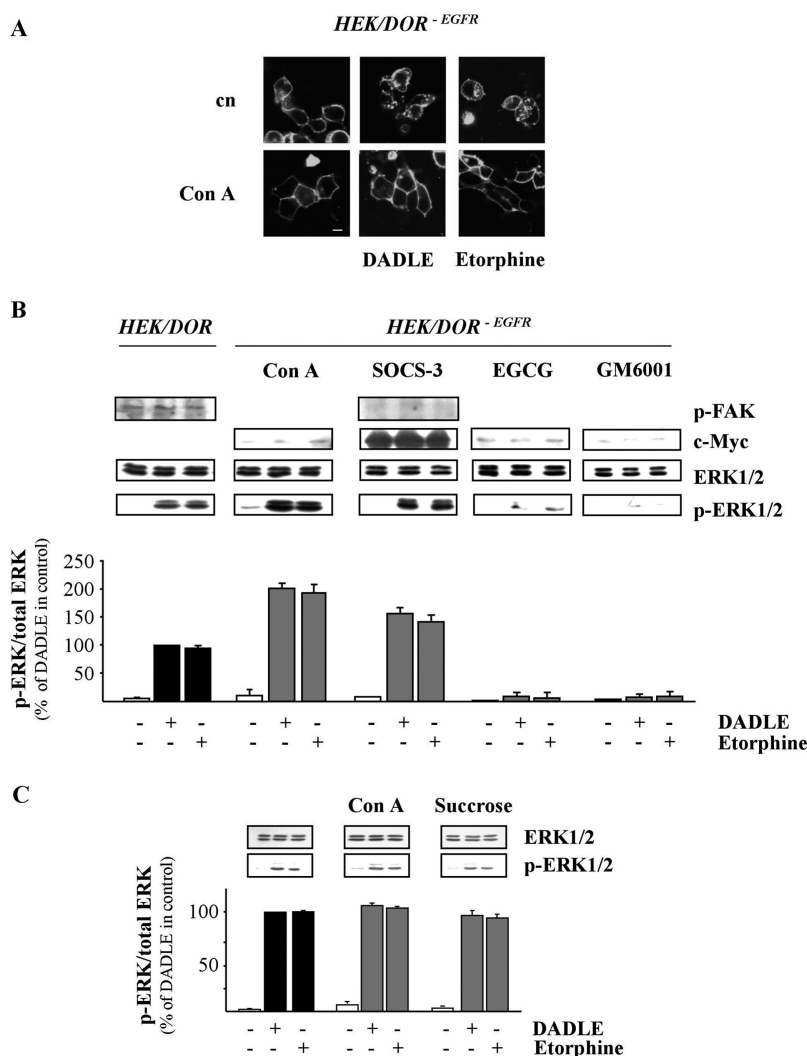


**Fig. 1.** Regulation of opioid-stimulated ERK1/2 activity by long-term EGF treatment. **A**, EGF receptors mediate opioid-stimulated ERK1/2 signaling in HEK/DOR but not in HEK/DOR<sup>-EGFR</sup> cells. HEK/DOR cells were continuously treated with EGF (0.1  $\mu$ g/ml; 18 h) to desensitize the EGF receptor. These cells were designated HEK/DOR<sup>-EGFR</sup>. Cells were then washed and equilibrated for 2 h in DMEM/0.1% FCS either in the absence or presence of EGF. When indicated, the EGF receptor blockers AG1478 (5  $\mu$ M) or CL-387,785 (1  $\mu$ M) were added at the end of the preincubation period (30 min). Cells were then stimulated for 5 min with 1  $\mu$ M DADLE, 0.1  $\mu$ M etorphine, and 10 ng/ml EGF before reactions were stopped and ERK1/2 activation was assessed by means of Western blotting using a phosphospecific anti-p42/44 antibody (p-ERK1/2). Overall ERK1/2 abundance was evaluated using a pan-reactive anti-p42/44 antibody (ERK1/2). Opioid-stimulated ERK1/2 activity is expressed as the ratio of phospho-ERK1/2 to ERK1/2 immunoreactivity, which was normalized to the maximum effect of DADLE in HEK/DOR cells (set to 100%). Data shown are the mean values  $\pm$  S.D. from at least  $n = 3$  independent experiments. **B**, down-regulation of EGF receptors by long-term EGF treatment. HEK/HA-DOR cells were continuously treated with EGF (0.1  $\mu$ g/ml; 18 h) before membranes were prepared and relative EGF receptor, and DOR abundances were determined by Western blot. Equal protein loading was verified by staining of the blots with an anti-G protein  $\beta$ 1/2 subunit antibody. **C**, effect of siRNA-mediated EGF receptor knockdown on EGF and DOR-mediated ERK1/2 signaling. HEK/DOR cells were transfected with mixtures of three control or EGF receptor-specific siRNAs and allowed to grow overnight. After serum-starvation for 2 h, cells were pretreated with AG1478 (5  $\mu$ M) and CL-387,785 (1  $\mu$ M) for 30 min. Subsequently, cells were incubated for 5 min with DADLE (1  $\mu$ M), etorphine (0.1  $\mu$ M), and EGF (10 ng/ml), and examined for EGF receptor abundance and ERK1/2 phosphorylation by Western blotting as described above. The immunoblots shown are representative of three independent experiments yielding qualitative similar results.

step supernatant transfer approach was developed. This method uses the conditioned media of control and opioid-stimulated HEK/DOR and HEK/DOR<sup>-EGFR</sup> donor cells to stimulate ERK1/2 activation in HEK293 *wt* and continuously EGF-treated HEK293<sup>-EGFR</sup> acceptor cells (Fig. 3A). Incubation of HEK293 *wt* acceptor cells for 5 min with media collected from nonstimulated HEK/DOR donor cells produced only basal levels of ERK1/2 activity. After stimulation of the cells with DADLE (1  $\mu$ M; 5 min), the conditioned media markedly enhanced ERK1/2 phosphorylation in HEK293 *wt* acceptor cells (Fig. 3B). Because of the lack of endogenous opioid receptors, stimulation of mitogenic signaling in HEK293 *wt* acceptor cells must be confined to bioactive compounds released into the supernatant of HEK/DOR donor cells rather than to DADLE itself. In HEK/DOR donor cells, the supernatant obtained from DADLE-stimulated HEK/DOR<sup>-EGFR</sup> donor cells had only a little stimulatory effect on ERK1/2 activity in HEK293 *wt* acceptor cells (Fig. 3B). These results indicate that DOR activation in HEK/DOR<sup>-EGFR</sup> donor cells seems to attenuate the proteolytic release of functionally active compounds from the cell surface. Completely opposite results were obtained when conditioned media from DADLE-stimulated HEK/DOR and HEK/DOR<sup>-EGFR</sup> donor cells were applied to continuously EGF-treated HEK293<sup>-EGFR</sup> acceptor cells. Here, media from opioid-stimulated HEK/DOR

“donor” cells had only little effect, whereas those from opioid-stimulated HEK/DOR<sup>-EGFR</sup> cells strongly activated ERK1/2 signaling in HEK293<sup>-EGFR</sup> acceptor cells. Similar results were obtained when conditioned media from DADLE-treated HEK/DOR<sup>-EGFR</sup>, but not from HEK/DOR donor cells, were applied to CHO-K1 acceptor cells lacking endogenous EGF receptors (Sturla et al., 2005). These results indicate that in HEK/DOR<sup>-EGFR</sup> donor cells DOR activation induces the release of an ERK1/2 activator other than from the family of EGF-like ligands whose cognate receptor is already present in CHO-K1 or induced in HEK/DOR cells by long-term EGF treatment.

**DOR-Mediated ERK1/2 Signaling in HEK/DOR<sup>-EGFR</sup> Cells Involves Transactivation of IGF-1 Receptors.** To identify the alternative RTK system transactivated by opioids in HEK/DOR cells after long-term EGF treatment, HEK293<sup>-EGFR</sup> acceptor cells were preincubated with small-molecule inhibitors for several RTKs and analyzed for stimulation of ERK1/2 activity by conditioned media from DADLE-treated HEK/DOR<sup>-EGFR</sup> donor cells. As shown in Fig. 4A, the PDGF receptor-selective inhibitor AG1295 (Kovalenko et al., 1994) failed to interfere with supernatant-induced ERK1/2 stimulation. In contrast, pretreatment of HEK293<sup>-EGFR</sup> acceptor cells with the selective IGF-1 receptor blockers AG1024 (Wen et al., 2001) and AG538 (Blum et al., 2000) completely attenuated mitogenic signaling stimu-



**Fig. 2.** Stimulation of ERK1/2 activity by opioids in HEK/DOR<sup>-EGFR</sup> cells is mediated by MMPs. **A**, long-term EGF treatment does not affect opioid-induced DOR internalization. HEK293 cells were transiently transfected to express the EGFP-tagged DOR (green). At the end of EGF (0.1  $\mu$ g/ml) pretreatment, cells were kept for 30 min either in the absence (cn) or presence of Con A (250  $\mu$ g/ml) before DOR internalization was induced by the addition of 1  $\mu$ M DADLE and 0.1  $\mu$ M etorphine for 60 min. Whereas short-term exposure to opioids results in a punctuate accumulation of the EGFP-tagged receptor in the cytosol (top), the fluorescence remains at the plasma membrane in the presence of Con A (bottom). The images shown are representative of at least three independent experiments (scale bar, 10  $\mu$ m). **B**, identification of the pathway mediating opioid-induced ERK1/2 activation after long-term EGF treatment. Naive and transiently c-Myc-SOCS-3-transfected HEK/DOR cells were continuously exposed to EGF (0.1  $\mu$ g/ml; 18 h) for EGF receptor down-regulation (HEK/DOR<sup>-EGFR</sup>). Cells were washed and equilibrated for 2 h to serum-free conditions in the continued presence of EGF. When indicated, Con A (250  $\mu$ g/ml; 30 min), EGCG (50  $\mu$ M; 2 h), and GM6001 (2  $\mu$ M; 1 h) were added to the incubation medium to block receptor endocytosis and MMP activity, respectively. Mitogenic signaling was stimulated for 5 min by the addition of DADLE (1  $\mu$ M) and etorphine (0.1  $\mu$ M) before ERK1/2 activation was determined by Western blotting as in Fig. 1A. **C**, opioid-stimulated ERK1/2 signaling in CHO-K1 cells is not mediated by DOR internalization. CHO-K1 cells were transfected to transiently express the DOR and incubated with Con A and hypertonic sucrose as above to block receptor endocytosis. Thereafter, cells were examined for DADLE and etorphine-induced ERK1/2 phosphorylation. Opioid-stimulated ERK1/2 activity is expressed as the ratio of phospho-ERK1/2 to ERK1/2 immunoreactivity. Data were normalized to the maximum effect of DADLE in CHO-K1 cells, which served as the control (set to 100%). Data shown represent the mean values  $\pm$  S.D. from at least  $n = 3$  independent experiments.

lated by conditioned medium from DADLE-activated HEK/DOR<sup>-EGFR</sup> "donor" cells. The EGF receptor blocker AG1478 had no effect on ERK1/2 activation in this system. Similar results were obtained when the conditioned media from opioid-treated HEK/DOR<sup>-EGFR</sup> donor cells were applied to CHO-K1 acceptor cells. Again, only the IGF-1 receptor blockers AG1024 and AG538 were able to interfere with ERK1/2 activation induced by conditioned media from DADLE-stimulated HEK/DOR<sup>-EGFR</sup> cells, whereas pretreatment with the PDGF receptor blocker AG1295 and the EGF receptor inhibitor AG1478 was ineffective (Fig. 4B). Together, these results suggest that the ligand released in DADLE-stimulated HEK/DOR<sup>-EGFR</sup> cells most likely transactivates the IGF-1 receptor.

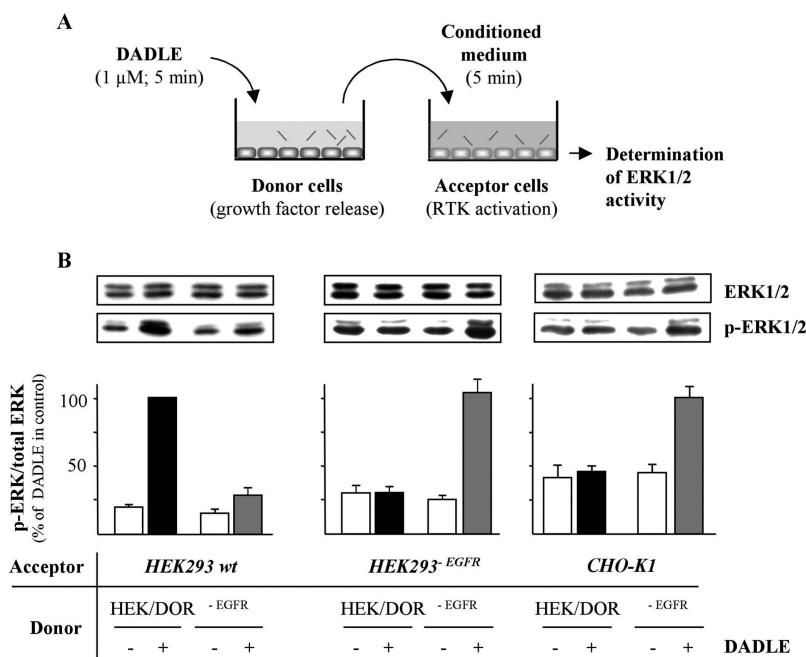
To verify that IGF-1 receptors indeed mediate opioid-induced stimulation of ERK1/2 signaling in HEK293<sup>-EGFR</sup> and CHO-K1 cells, we made an attempt to down-regulate the receptor by siRNA-mediated gene silencing before the cells were stimulated with conditioned media from HEK/DOR<sup>-EGFR</sup> cells. However, as shown in a control experiment, transfection of the cells with siRNAs specific for the IGF-1 receptor failed to prevent IGF-1 (10 ng/ml, 5 min)-stimulated ERK1/2 phosphorylation, although IGF-1 receptor immunoreactivity almost completely disappeared from the plasma membrane as assessed by Western blot analysis (Fig. 4C). Therefore, we directly examined IGF-1 receptor phosphorylation after DOR activation in HEK/DOR and HEK/DOR<sup>-EGFR</sup> cells. As shown in Fig. 4D, long-term EGF treatment facilitates the ability of DADLE (1  $\mu$ M, 5 min) and etorphine (0.1  $\mu$ M, 5 min) to stimulate IGF-1 receptor phosphorylation, an effect that is absent in untreated HEK/DOR cells. In addition, opioid-induced ERK1/2 stimulation in HEK/DOR<sup>-EGFR</sup> cells is blocked by preincubation with the IGF-1 receptor blockers AG1024 and AG538. These results implicate that in HEK/DOR<sup>-EGFR</sup> cells DOR-mediated ERK1/2 signaling is mediated by transactivation of IGF-1 receptors.

**Long-Term EGF Treatment of HEK/DOR Cells Alters the Spectrum of Opioid-Released Growth Factors.** The switch from an EGF to an IGF-1 receptor-dependent mecha-

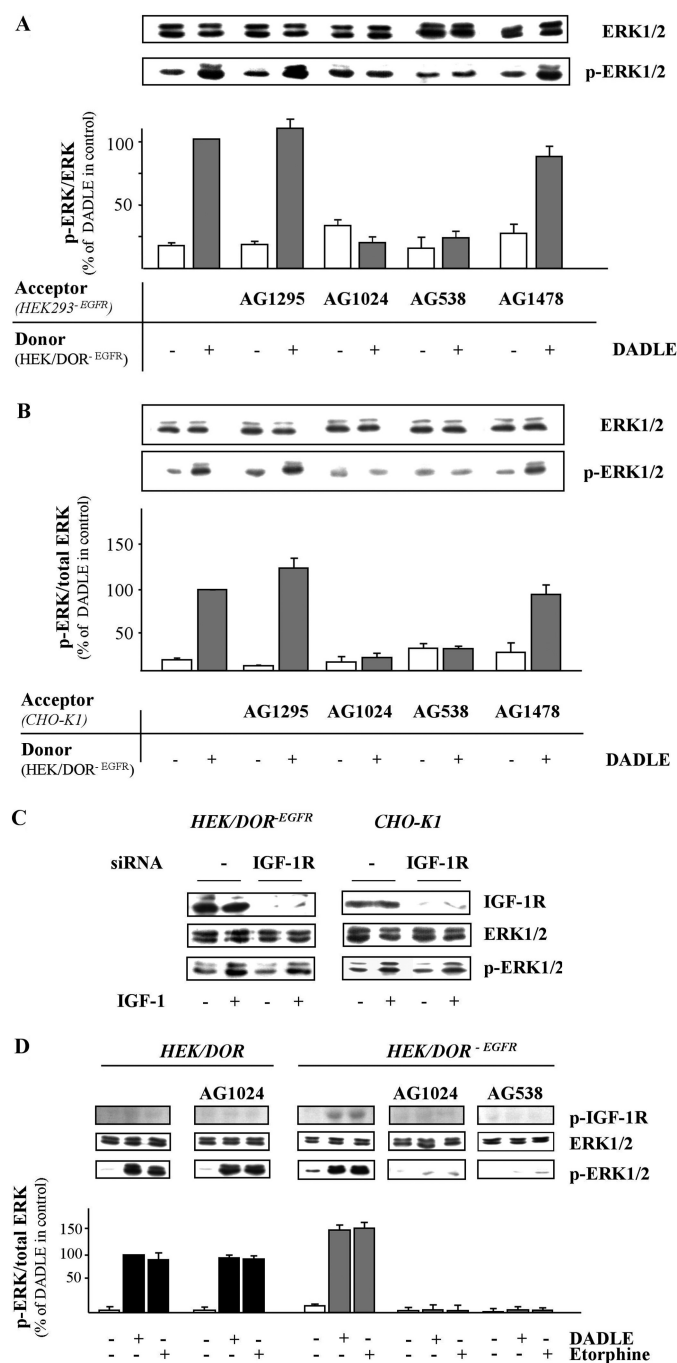
nism in opioid-stimulated ERK1/2 signaling might be mediated by an increased expression or functional activity of IGF-1 receptors and/or a change in the soluble factors released into the supernatant. To discriminate between these possibilities, we first investigated possible changes in the expression level of the IGF-1 receptor. However, as shown by Western blot analysis using an overall reactive and a phospho(Tyr1135/1136)-specific IGF-1 receptor  $\beta$ -chain antibody, there was no detectable change in both the relative abundance and the autophosphorylation state after long-term EGF treatment (Fig. 5A).

To identify possible changes in the proteolytic release of growth factors from the cell surface after long-term EGF treatment, the incubation media from control and DADLE-stimulated HEK/DOR and HEK/DOR<sup>-EGFR</sup> cells were subjected to microfluidic electrophoresis using the Agilent 2100 Bioanalyzer. This approach allows the resolution and sensitive detection of polypeptides in biological fluids. As shown in Fig. 5B, serum-free incubation media of untreated HEK/DOR cells contain only low concentrations of a single peptide of unknown identity. After stimulation of the cells with DADLE (1  $\mu$ M; 5 min), large amounts of a peptide comigrating with the EGF receptor ligand HB-EGF applied as a standard appear in the supernatant. Long-term EGF treatment produces fundamental changes the spectrum of peptides released. Under basal conditions, two low-abundance peptides not related to that observed in untreated HEK/DOR cells are present. Although stimulation of the cells with DADLE induces the release of comparable amounts of HB-EGF as in HEK/DOR cells, two additional peptides are released into the conditioned media of HEK/DOR<sup>-EGFR</sup> cells, one of which comigrates along with the monomeric form of recombinant IGF-1 (Peters et al., 1985). These results demonstrate that DOR activation in HEK/DOR<sup>-EGFR</sup> cells not only results in the shedding of HB-EGF but also of an IGF-1-like ligand.

**IGF-1 Receptors Mediate ERK1/2 Signaling in Continuously NGF-Treated NG108-15 Cells.** To evaluate whether the ability of opioids to use different RTK systems







**Fig. 4.** IGF-1 receptors mediate opioid-induced ERK1/2 activation in HEK/DOR-EGFR cells. **A** and **B**, identification of the RTK mediating DOR-stimulated ERK1/2 signaling in continuously EGF-treated HEK/DOR cells. Conditioned media from DADLE (1  $\mu$ M; 5 min)-treated or untreated HEK/DOR-EGFR donor cells were collected and used to stimulate ERK1/2 activity in EGF receptor-deficient HEK293-EGFR and CHO-K1 acceptor cells as described in Fig. 3B. Before the addition of conditioned media, acceptor cells were pretreated for 30 min with tyrosine kinase inhibitors AG1295, AG1024, AG538, and AG1478 to inactivate PDGF, IGF-1, and EGF receptors, respectively. Note that inhibition of IGF-1 receptors in HEK293-EGFR and in CHO-K1 acceptor cells blocks ERK1/2 activation by conditioned medium of opioid-stimulated HEK/DOR-EGFR cells. **C**, effect of IGF-1 receptor gene silencing on IGF-1 receptor abundance and ERK1/2 signaling. Cells were transfected with mixtures of three control (-) and IGF-1 receptor-specific siRNAs and allowed to grow overnight either in the absence (CHO-K1) or presence of EGF (0.1  $\mu$ M; 18 h; HEK/DOR-EGFR). Cells were stimulated with IGF-1 (10 ng/ml; 5 min) and analyzed for IGF-1 receptor expression and total and phospho-ERK1/2 immunoreactivity. **D**, opioid-induced stimulation of ERK1/2

for ERK1/2 signaling represents a common phenomenon, the same experiments were performed on endogenously DOR-expressing NG108-15 hybrid cells. In these cells, opioid-induced ERK1/2 signaling is mediated by transactivation of TrkA receptors (Eisinger and Ammer, 2008b). Preincubation of the cells with the IGF-1 receptor blocker AG1024 failed to attenuate DADLE and etorphine-stimulated ERK1/2 phosphorylation (Fig. 6). Although long-term exposure of NG108-15 cells to NGF (100 ng/ml; 18h) completely attenuated TrkA-induced stimulation of ERK1/2, it had no effect on DOR-mediated ERK1/2 activation. Because AG1024, but not the TrkA receptor inhibitor AG879 (Ohmichi et al., 1993), prevented DOR-mediated ERK1/2 stimulation in NG108-15<sup>-TrkA</sup> cells, long-term NGF treatment seems to induce a similar switch in opioid-induced ERK1/2 activation from a TrkA to an IGF-1 receptor-dependent mechanism.

## Discussion

The present study demonstrates that long-term EGF treatment of HEK/DOR cells results in a switch in opioid-induced ERK1/2 signaling from an EGF to an IGF-1 receptor-dependent pathway. The underlying regulatory mechanism involves down-regulation and functional desensitization of EGF receptors and the release of an IGF-1 like peptide from the plasma membrane, which in turn mediates opioid-induced ERK1/2 signaling via transactivation of IGF-1 receptors. A similar switch from a TrkA to an IGF-1 receptor-mediated mechanism in opioid-stimulated ERK1/2 signaling was found for endogenously DOR-expressing NG108-15 hybrid cells after long-term NGF treatment. These results demonstrate that opioids are able to use multiple alternative RTK systems within a single cell to maintain persistent mitogenic signaling.

HEK293 cells are frequently used as a model system to study GPCR-mediated ERK1/2 signaling (Voss et al., 1999; Luttrell, 2005). Besides transactivation of EGF receptors, these cells also support additional intracellular signal transduction mechanisms coupled to ERK1/2 signaling, including agonist-induced receptor internalization (Pierce et al., 2000) and activation of pp125FAK (Della Rocca et al., 1999). With respect to opioids, we could demonstrate that the stable opioid peptide agonist DADLE and the cell membrane-permeable alkaloid etorphine both stimulate ERK1/2 signaling via a common EGF receptor-dependent pathway (Eisinger and Ammer, 2008b). Here, we show that functional inactivation of the EGF receptor system in stably DOR-expressing HEK293 cells does not abrogate but rather redirect opioid-induced ERK1/2 signaling via the IGF-1 receptor system. Most interestingly, long-term EGF treatment rather than siRNA-mediated gene silencing turned out an essential prerequisite to detect the changes in opioid-induced ERK1/2 signaling. Although the latter approach successfully decreased EGF receptor abundance lower than the detection limit in Western blot, it failed to abrogate EGF (10 ng/ml)-

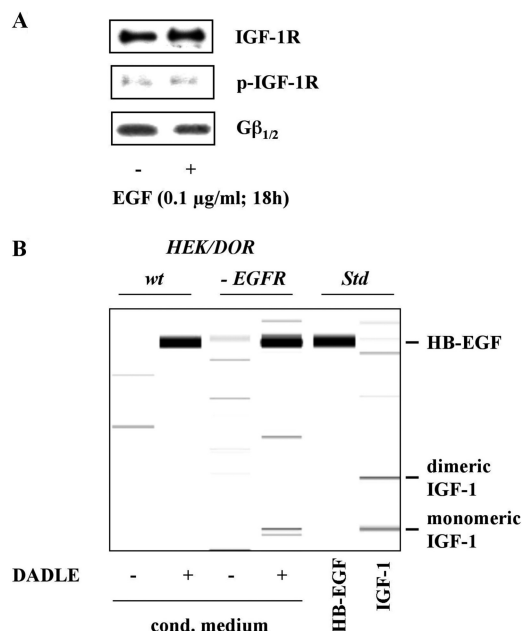
activity in HEK/DOR-EGFR cells is mediated by transactivation of IGF-1 receptors. HEK/DOR and HEK/DOR-EGFR cells were pretreated with the IGF-1 receptor blockers AG1024 and/or AG538 during the last 30 min of the equilibration period to serum-free conditions. Stimulation of IGF-1 receptor phosphorylation and ERK1/2 activity was determined for 5 min in the presence of 1  $\mu$ M DADLE and 0.1  $\mu$ M etorphine before reactions were stopped and proteins were subjected to Western blot analysis using phosphospecific anti-p42/44 and anti-IGF-1R antibodies.

induced ERK1/2 signaling. Incomplete loss of EGF receptor function has been already reported previously for siRNA-treated MCF-7 cells (Santen et al., 2009), supporting the notion that long-term EGF treatment not only down-regulates but also functionally desensitizes residual EGF receptors (Beguinot et al., 1984). Besides inactivation of EGF receptor function, siRNA-mediated knockdown of EGF receptors also failed to redirect DOR-induced ERK1/2 signaling to an alternative RTK pathway. These results suggest that long-term EGF treatment must produce additional intracel-

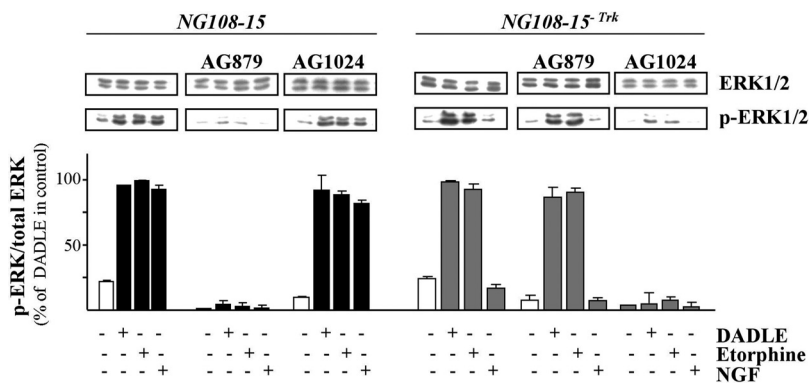
lular adaptations that facilitate the switch in DOR-mediated ERK1/2 signaling from an EGF- to an IGF-1 receptor-dependent mechanism. Such a heterologous long-term EGF effect seems plausible and might explain the increase in DOR abundance and sensitization of opioid-stimulated ERK1/2 signaling in HEK/DOR<sup>-EGFR</sup> cells. A similar regulation of G protein-coupled receptors has been described previously for in PC12 cells, in which long-term EGF treatment increased muscarinic acetylcholine receptor abundance by mRNA stabilization (Lee and Malek, 1998).

The newly acquired signal transduction pathway by which opioids bring approximately ERK1/2 activation after long-term EGF treatment involves neither agonist-induced DOR internalization nor activation of pp125FAK. Experiments with two structurally unrelated MMP blockers rather suggest the establishment of an alternative paracrine mechanism that replaces opioid-induced transactivation of EGF receptors in HEK/DOR<sup>-EGFR</sup> cells. MMPs are zinc-dependent endoproteases that regulate a number of cellular processes by the controlled proteolytic cleavage of membrane-bound growth factors (McCawley and Matrisian, 2001). Indeed, the findings of our two-step supernatant transfer approach clearly demonstrate that conditioned media from opioid-stimulated HEK/DOR<sup>-EGFR</sup> but not from untreated HEK/DOR donor cells are able to induce ERK1/2 phosphorylation in HEK293<sup>-EGFR</sup> and EGF receptor-deficient CHO-K1 acceptor cells. Because HEK293 *wt* and CHO-K1 cells lack endogenous opioid receptors (Carboni et al., 1997; Schulz et al., 2004), stimulation of mitogenic signaling must be accomplished by the presence of a soluble factor released into the supernatant of HEK/DOR<sup>-EGFR</sup> donor cells in response to DOR activation.

GPCRs are able to mediate ERK1/2 signaling via transactivation of multiple RTKs, including the PDGF and IGF-1 receptor (Della Rocca et al., 1999). Although HEK293 *wt* and CHO-K1 cells carry functional active PDGF receptors that are coupled to the Ras/Raf/ERK1/2 module (Oak et al., 2001), ERK1/2 activation was not affected by the PDGF receptor-specific inhibitor AG1295 (Kovalenko et al., 1994) in our experiments. Because the release of PDGF from plasma membranes is mediated by convertase-dependent ectodomain shedding (Siegfried et al., 2005), which is insensitive to broad-spectrum MMP blockers (Antoine et al., 2009), the present study indicates that PDGF receptors are not involved in the mechanism of opioid-induced ERK1/2 signaling in HEK/DOR<sup>-EGFR</sup> cells. Instead, several lines of evidence suggest that functional inactivation of EGF receptors redirects opioid-stimulated ERK1/2 activation via the transactivation of IGF-1 receptors: 1) pretreatment of HEK293<sup>-EGFR</sup> and CHO-K1 acceptor cells



**Fig. 5.** Long-term EGF treatment of HEK/DOR cells alters the spectrum of opioid-released growth factors. A, long-term EGF treatment fails to affect overall IGF-1 receptor abundance and phosphorylation. Membranes (IGF-1R,  $G\beta_{1/2}$ ) and whole-cell solubilizates (phospho-IGF-1R) from naive and continuously EGF-treated HEK/HA-DOR cells were electrophoresed, transferred onto Immobilon-P membranes, and stained with antibodies against the regulatory subunit of the human IGF-1 receptor, the autophosphorylation sites of the IGF-1 receptor, and the G protein  $\beta_{1/2}$  subunit as described in Fig. 1B. B, analysis of the soluble factors released by DOR activation. Serum-free media of DADLE-stimulated and untreated HEK/DOR and HEK/DOR<sup>-EGFR</sup> "donor" cells were precipitated by trichloroacetic acid and subjected to microfluidic electrophoresis under denaturing conditions. HB-EGF (2 ng) and IGF-1 (6 ng) served as the standards and were run in parallel on the same chips. Conditioned media from DADLE-treated HEK/DOR and HEK/DOR<sup>-EGFR</sup> cells contain a peptide displaying the same electrophoretic mobility than HB-EGF. A soluble ligand comigrating with the monomeric form of IGF-1 is only found in HEK/DOR<sup>-EGFR</sup> after DOR activation. The experiment shown is representative of three individual experiments producing qualitatively similar results.



**Fig. 6.** Effect of long-term NGF treatment on DOR-mediated ERK1/2 signaling in NG108-15 cells. NG108-15 cells were incubated for 18 h in the absence or presence of NGF (100 ng/ml) to desensitize TrkA receptors (NG108-15<sup>-Trk</sup>). Cells were equilibrated to serum-free conditions for 2 h and subsequently treated with DADLE (1  $\mu$ M), etorphine (0.1  $\mu$ M), or NGF (100 ng/ml) for an additional 5 min. When indicated, the TrkA and IGF-1 receptor blockers AG879 (100  $\mu$ M) and AG1024 (10  $\mu$ M) were added during the last 30 min of the equilibration period. ERK1/2 phosphorylation and overall ERK1/2 abundance was determined by Western blot as in Fig. 1A. ERK1/2 activation is expressed as the ratio of pERK1/2 to ERK1/2 immunoreactivity, which was normalized to the effect of DADLE in the controls (set to 100%). All results are the means  $\pm$  S.D. values from  $n = 3$  independent experiments.



with the IGF-1 receptor inhibitors AG1024 (Wen et al., 2001) and AG538 (Blum et al., 2000) blocks ERK1/2 activation by conditioned media obtained from opioid-stimulated HEK/DOR<sup>-EGFR</sup> cells; 2) DOR activation is associated with IGF-1 receptor phosphorylation in HEK/DOR<sup>-EGFR</sup> cells, an effect that is sensitive to AG1024 and AG538; and 3) in addition to HB-EGF, DOR activation triggers the release of an IGF-1-like peptide into the culture medium from continuously EGF-treated HEK/DOR cells. Such a switch in the RTK system transactivated by opioids may be explained by the presence of MMP isoforms 2 and 9 in HEK/DOR cells (Schulz et al., 2004), which are rather nonspecific and cleave both HB-EGF and IGF-1 from their respective precursors and binding proteins (Meng et al., 2008). In addition, long-term EGF treatment has been reported previously to induce the synthesis and the release of IGF-1-like peptides from adult rat hepatocytes, an effect that is accompanied with an increased expression of both IGF-1 and IGF binding protein IGFBP-1 mRNA (Barreca et al., 1992).

The continued shedding of HB-EGF by DOR activation in HEK/DOR<sup>-EGFR</sup> donor cells contrasts the finding that conditioned medium from these cells fails to stimulate ERK1/2 activity in HEK293 *wt* acceptor cells that should carry functionally active EGF receptors. One plausible explanation for this discrepancy would be the presence of a negative regulator of HB-EGF activity in the conditioned medium of HEK/DOR<sup>-EGFR</sup> donor cells. Indeed, long-term EGF treatment has been reported to induce shedding of the membrane-associated heparin sulfate proteoglycan Syndecan-1 into the extracellular milieu (Subramanian et al., 1997), which acts as a soluble scavenger for HB-EGF that inhibits binding to EGF receptors (Bernfield et al., 1999).

The question remains of why the supernatant from DADLE-stimulated HEK/DOR<sup>-EGFR</sup> donor cells fails to induce ERK1/2 activation in HEK293 *wt* acceptor cells, although HEK293 *wt* cells carry functional IGF-1 receptors (El-Shewy et al., 2004). Because long-term EGF treatment failed to identify a major increase in the quantity of IGF-1 receptors in membranes from HEK/DOR<sup>-EGFR</sup> cells, a functional mechanism must apply. For this, we analyzed IGF-1 receptor autophosphorylation in response to long-term EGF treatment, which has been shown previously to prolong the growth of EGF-treated BALB/c3T3 cells coexpressing the EGF and IGF-1 receptors (Pietrzkowski et al., 1992). However, our experiments failed to identify a comparable increase in IGF-1 receptor autophosphorylation after long-term EGF treatment in HEK/DOR<sup>-EGFR</sup> cells. These results suggest that long-term EGF treatment might increase the sensitivity of IGF-1 receptors to its ligand by down-regulation of its cognate inhibitor protein IGFBP-3 (Takaoka et al., 2006). Such a functional mechanism could explain why DOR activation selectively stimulates IGF-1 receptor phosphorylation in HEK/DOR<sup>-EGFR</sup> but not in HEK/DOR cells.

In conclusion, the present study demonstrates that DOR-mediated ERK1/2 signaling is not restricted to transactivation of EGF receptors in HEK/DOR or TrkA receptors in NG108-15 hybrid cells. After functional desensitization of the dominant RTK systems in these cell lines, opioid-induced stimulation of ERK1/2 activity is redirected via the IGF-1 receptor system. The switch in the RTK system used by opioids to bring about ERK1/2 signaling is in line with the observation that the activity state of the predominant RTK in a given cellular setting determines the functional activity of

the IGF-1 receptor system (Pietrzkowski et al., 1992; Roudabush et al., 2000). Cross-regulation of RTK systems is of substantial physiological significance in the developing and adult brain. Whereas EGF and TrkA receptors regulate the embryonic development of different neuronal cells, the IGF-1 receptor system controls adult neurogenesis (Kato et al., 1995; Lewin and Barde, 1996). In this respect, the ability of opioids to transactivate each of these RTK systems as described herein might contribute to opioid modulation of neurogenesis during fetal development and in the adult brain (Sargeant et al., 2008). Because the switch in opioid-induced RTK signaling largely relies on the ability of MMPs to cleave a broad spectrum of growth factor precursors, the present study raises the intriguing possibility that opioid receptors might serve as potential drug targets to interfere with the biological functions of a number of RTK systems in a wide variety of cell lines and tissues.

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#### Authorship Contributions

*Participated in research design:* Eisinger.

*Conducted experiments:* Eisinger and Ammer.

*Performed data analysis:* Eisinger.

*Wrote or contributed to the writing of the manuscript:* Eisinger and Ammer.

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