Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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$GABA_BR$-Induced EGFR Transactivation Promotes Migration of Human Prostate Cancer Cells

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Running title: EGFR transactivation induced by $GABA_BR$

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Number of

Text pages: 41

Tables: 0

Figures: 9

References: 70

Words in

abstract: 206

introduction: 661

discussion: 1145

List of nonstandard abbreviations

AR, amphiregulin; CGNs, cerebellar granular neurons; CM, conditioned medium; EGF, epithelial growth factor; EGFR, EGF receptor; ELISA, enzyme-linked immuno-sorbent assay; ERK1/2, extracellular-signal regulated kinase 1/2; HB-EGF,

heparin-binding EGF; IGF-1R, insulin-like growth factor receptor 1; $GABA_BR$, metabotropic $GABA_B$ receptor; GPCRs, G-protein coupled receptors; HRP, horse radish peroxidase; MMPs, matrix metalloproteinases; PAMs, positive allosteric modulators; NCE, non-clathrin endocytosis; PCa, prostate cancer; PDGFR, platelet-derived growth factor receptor; PTX, pertussis toxin; RT, room temperature; RTK, receptor tyrosine kinase; SFM, serum free medium; TCL, total cell lysate; TGF-α, transforming growth factor α; TMB, tetramethylbenzidine.

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Abstract

G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) act in concert to regulate cell growth, proliferation, survival, and migration. Metabotropic $GABA_B$ receptor ($GABA_BR$) is the GPCR for the main inhibitory neurotransmitter GABA in the central nervous system. Increased expression of $GABA_BR$ has been detected in human cancer tissues and cancer cell lines, but the role of $GABA_BR$ in these cells is controversial and the underlying mechanism remains poorly understood. Here, we investigated whether $GABA_BR$ hijacks RTK signaling to modulate the fates of human prostate cancer cells. RTKs array analysis revealed that $GABA_BR$ specific agonist, baclofen, selectively induced the transactivation of EGFR in PC-3 cells. EGFR transactivation resulted in the activation of ERK1/2 by a mechanism that is dependent on $G_{i/o}$ protein and that requires matrix metalloproteinases (MMPs) mediated pro-ligand shedding. Positive allosteric modulators (PAMs) of $GABA_BR$, such as CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion through EGFR signaling. In summary, our observations demonstrated that $GABA_BR$ transactivated EGFR in a ligand-dependent mechanism to promote prostate cancer cell migration and invasion, thus providing new insights into developing novel strategy for prostate cancer treatment by targeting neurotransmitter signaling.

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Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide (Torre et al., 2015). Currently, few therapeutic options are available for patients with advanced PCa. Tyrosine kinase inhibitors (TKIs), being effective in some human malignancies (Rask-Andersen et al., 2014), have been used as single agents or in combination with chemotherapy in clinical trials in patients with castration-resistant PCa; however, the results were not promising (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014; Modena et al., 2016). Therefore, new mechanism-based inhibitors need to be developed to treat PCa patients. Since neurotransmitters have modulatory effects on tumor cells, the potential roles of receptors for neurotransmitters in tumors have attracted more and more research interests (Schuller, 2008a).

GABA is a main inhibitory neurotransmitter in the vertebrate central nervous system. Metabotropic $GABA_B$ receptor ($GABA_BR$), a G protein-coupled receptor (GPCR) family member, is an obligatory heterodimer composed of $GABA_{B1}$ and $GABA_{B2}$ subunits. The extracellular domain of $GABA_{B1}$ subunit has a ligand binding site for GABA, agonists, or antagonists. Whereas the extracellular domain of $GABA_{B2}$ subunit lacks the ligand binding capacities, $GABA_{B2}$ subunit is responsible for $G_{i/o}$ protein coupling. It has been shown that specific allosteric modulators bind to $GABA_{B2}$ subunit transmembrane domain (Bettler et al., 2004; Pinard et al., 2010).

Accumulated evidence suggests that $GABA_BR$ is implicated in human cancers. The level of $GABA_BR$ in thyroid and breast cancer specimens is positively correlated with tumor malignancy (Roberts et al., 2009; Jiang et al., 2012); moreover, $GABA_BR$ increases the metastasis of mouse breast 4T1 cancer cells *in vivo* (Zhang et al., 2014). In contrast, the level $GABA_{B1}$ subunit in cholangiocarcinoma tissues is negatively

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correlated with the degree of cell differentiation, local invasion, and lymph node metastasis (Huang et al., 2013). Upregulated expression of $GABA_{B2}$ subunit is detected in female lung cancer patients with better outcome (Zhang et al., 2013), while the level of $GABA_BR$ has no obvious correlation with the pathological features of human gastric cancer (Zhu et al., 2004). *In vitro* studies reveal that $GABA_BR$ agonists may inhibit (Fava et al., 2005; Wang et al., 2008; Huang et al., 2013; Zhang et al., 2013; Shu et al., 2016) or dispaly no effect (Abdul et al., 2008; Lodewyks et al., 2011; Zhang et al., 2014) on the proliferation of cancer cells. Activation of $GABA_BR$ may promote (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014), suppress (Fava et al., 2005; Schuller et al., 2008b; 2008c; Lodewyks et al., 2011), or have no influences (Joseph et al., 2002; Drell et al., 2003; Chen et al., 2012) on the migration and/or invasion of cancer cells. These observations suggest that the roles of $GABA_BR$ in cancer cell proliferation, migration, and tumor malignancy are controversial.

It has been well documented that GPCRs induce the transactivation of various receptor tyrosine kinases (RTKs) including epithelial growth factor receptor (EGFR) family members, insulin-like growth factor receptor 1 (IGF-1R), fibroblast growth factor receptor 1, platelet-derived growth factor receptor, vascular endothelial growth factor receptor 2, TrkA/B, and c-Met (Wetzker and Böhmer, 2003; Tu et al., 2010; Cattaneo et al., 2014; Ryu et al., 2014). We have reported previously that specific activation of $GABA_BR$ induced transactivation of IGF-1R in neurons (Tu et al., 2010; Lin et al., 2012). To investigate whether $GABA_BR$ hijacks RTK signaling to modulate the fates of PCa cells, RTK array analysis was performed. The results showed that a specific $GABA_BR$ agonist, baclofen, selectively induced transactivation of EGFR at multiple sites (Y845, Y1045, Y1068 and Y1086) in PC-3 cells, which was dependent on $G_{i/o}$ protein and pro-ligand shedding mediated by matrix metalloproteases (MMPs).

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Interestingly, positive allosteric modulators (PAMs) of $GABA_BR$, including CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion, which were dependent on EGFR transactivation. These observations suggest that $GABA_BR$ mediated EGFR signaling might be the potential targets in the treatment of advanced PCa.

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Materials and Methods

Antibodies and Reagents

Primary antibodies including pERK1/2 (T202/Y204), ERK1/2, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), EGFR, β-actin, EGFR antibody-conjugated sepharose-beads, and horse radish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphotyrosine antibody 4G10, 4G10-conjugated agarose-beads, and EGFR neutralizing antibody (LA1) were obtained from Millipore (Billerica, MA, USA).

Pertussis toxin (PTX) and MMPs inhibitor GM6001 (Ilomastat or Galardin) were purchased from Millipore (Billerica, MA, USA). $GABA_BR$ agonist baclofen, antagonist CGP54626, PAMs CGP7930, rac-BHFF, and GS39783 were obtained from Tocris Bioscience (Bristol, BS, UK). EGFR inhibitors Tarceva (Erlotinib) and Iressa (Gefitinib) were from Active Biochem (Hongkong, China).

Cell Culture

Human prostate cancer cell line PC-3 was purchased from ATCC. Cells were maintained in F-12 medium (Gibco, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C with 5% CO_2. Cells were split every 3-4 days and medium was replaced every 2 days.

Treatment of cells with various compounds

1.5×10^5 cells were seeded into 3.5 cm plates and grown in complete medium for 2 days at 37°C with 5% CO_2. Cells were then serum starved for 24 h followed by incubation with u7 一 serum free medium (SFM) for 30 min before being treated with

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various compounds as described below.

$GABA_BR$ agonist. Cells were incubated with 200 μM baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 min, or with 0, 50, 100, 150, 200, 250, and 300 μM of baclofen at 37°C for 5 min.

PAMs of $GABA_BR$. CGP7930, rac-BHFF, and GS39783 were solubilized in DMSO with stock concentrations of 100 mM, 100 mM, and 10 mM, respectively. Cells were treated with 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 min, or with various concentrations of CGP7930 (0, 3, 6, 12, 25, 50, and100 μM), rac-BHFF (0, 25, 50, 75, 100, 125, and 150 μM), or GS39783 (0, 0.6, 1.2, 2.5, 5, 10, and 20 μM) at 37°C for 10, 20, or 5 min, respectively. DMSO treated cells were used as control.

$GABA_BR$ antagonist. Cells were incubated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with 200 μM baclofen, 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 in the presence or absence of 20 μM CGP54626 at 37°C for 5, 10, 20, or 5 min, respectively.

$G_{i/o}$ inhibitor. Cells were serum starved for 24 h in the presence or absence of PTX (200 ng/ml) followed by treatment with 200 μM baclofen in the presence or absence of PTX at 37°C for 5 min.

EGFR inhibitors. Cells were incubated with 5 μM Tarceva, 5 μM Iressa, or 0.05% DMSO at 37°C for 8 h. At the end of incubation, cells were treated with 200 μM baclofen, 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 in the presence or absence of Tarceva or Iressa at 37°C for 5, 10, 20, and 5 min, respectively.

MMPs inhibitor. Cells were pre-treated with 10 μM GM6001 or 0.05% DMSO at 37°C for 1 h followed by incubation with 200 μM baclofen in the presence or absence of GM6001 at 37°C for 5 min.

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EGFR neutralizing antibody. Cells were incubated with or without 2 μg/ml of EGFR neutralizing antibody at 37°C for 1 h followed by treatment with 200 μM baclofen, 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 in the presence or absence of neutralizing antibody at 37°C for 5, 10, 20, and 5 min, respectively.

At the end of each treatment, cells were lyzed with RIPA buffer containing proteinase inhibitors cocktail on ice for 30 min, sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. The supernatants were transferred to new microtubes and stored at -20°C until use or subjected to SDS-PAGE immediately.

Receptor Tyrosine Kinase (RTK) Array Assay

Human RTK array was purchased from RayBiotech (Norcross, GA, USA). The experiment was performed according to manufacturer's instructions. Briefly, PC-3 cells grown in 10 cm plates were serum starved for 24 h followed by treatment with or without 200 μM baclofen at 37°C for 5 min. Cells were then washed quickly with cold PBS, and lyzed immediately with cold RIPA buffer on ice for 30 min. Total cell lysates (TCLs) were sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. Supernatants were diluted by blocking buffer in a final volume of 1.2 ml (300 μg/ml), and incubated with RTK array membranes, which were pre-incubated with blocking buffer overnight at 4°C. The membranes were washed three times (3 min each) with 2 ml of 1×Wash Buffer I at room temperature (RT) with shaking, carefully transferred to a plastic container, and washed with a minimum of 20 ml of 1×Wash Buffer I for three times (5 min each) followed by washing with a minimum of 20 ml of 1×Wash Buffer II for three times (5 min each). The membranes were transferred to an eight-well tray, incubated with 1.2 ml of diluted biotin-conjugated antibody at RT with gentle shaking for 2 h, followed by incubating with 1.5 ml of 1×HRP-conjugated streptavidin for 2 h at RT, washed with 1×Wash

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Buffer I and II as described previously. Membranes were developed by using enhanced chemiluminescence reagents (Thermo, China).

Immunoprecipitation

PC-3 cells grown in 10 cm plates were serum starved for 24 h in the presence or absence of PTX (200 ng/ml) followed by treatment with or without 200 μM baclofen at 37°C for 5 min. Serum starved PC-3 cells were also treated with or without 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 at 37°C for 10, 20, or 5 min, respectively. At the end of the treatment, cells were washed quickly with cold PBS, and lyzed immediately with cold RIPA buffer on ice for 30 min. TCLs were sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. The supernatants (500 μg) were mixed with 20 μl of 4G10-conjugated agarose-beads or EGFR antibody-conjugated sepharose-beads in a final volume of 500 μl, and incubated at 4°C overnight with continuous agitation. The beads were washed with RIPA buffer for five times (600 μl each) followed by PBS for 2 times, resuspended in 20 μl of 2×electrophoresis loading buffer, boiled for 5 min, and centrifuged at 12,000 rpm for 5 min. The supernatants were transferred to new microtubes and subjected to immunoblot analysis.

Immunoblot Analysis

Equal amounts of TCLs were resolved by 10% SDS-PAGE gels followed by membrane transfer. Membranes were blocked with TBS containing 5% non-fat dry milk and 0.1% Tween-20 for 2 h at RT, and incubated with primary antibodies against EGFR, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), ERK1/2, pERK1/2, or β-actin (1:2000-1:3000) overnight at 4°C with continuous agitation. The next day, the membranes were washed and probed with proper HRP-conjugated secondary

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antibodies (1:20,000-1:30,000) at RT for 2 h. Membranes were then washed and developed by using enhanced chemiluminescence reagents.

Enzyme Linked-Immunosorbent Assay (ELISA)

Human EGF, heparin-binging EGF (HB-EGF), amphiregulin (AR), and transforming growth factor-α (TGF-α) ELISA kits were purchased from BOSTER (Pleasanton, CA 94566, USA). Serum starved PC-3 cells seeded on 3.5 cm plates were treated with 200 μM baclofen at 37°C for 0, 5, 10, or 15 min, and conditioned media (CM) were transferred to 1.5 ml microtubes. 100 μl CM or standards were added into 96-well plates and incubated at 37°C for 90 min followed by extensive washing. 100 μl biotinylated antibodies were then added to the plates, incubated at 37°C for 60 min followed by 3 washes (1 min each). 100 μl avidin-biotin-peroxidase complex working solution was added to the plates except the tetramethylbenzidine (TMB) blank well and incubated at 37°C for 30 min followed by five washes (1-2 min each). 90 μl TMB developing reagent was added to the plate and incubated at 37°C in dark for 15-20 min. 100 μl TMB stop solution was added to the plates, and OD_{450} was measured in a microplate reader within 30 min. The experiments were performed in triplicates and repeated three times.

Transwell Assay

Modified Boyden chamber (Corning Costar, Rochester, NY, USA) containing a polycarbonate membrane filter (6.5 mm in diameter with pore size of 8 μm) was used in this assay. The upper chamber was coated with 70 μl of Matrigel (1 mg/ml; Corning) for invasion assay. 700 μl F-12 medium supplemented with 10% FBS was placed in the lower chamber. 200 μl of cell suspension (5×10^4 cells for migration, 1×10^5 cells for invasion) in the presence or absence of baclofen, CGP54626, CGP7930, or EGFR neutralizing antibody was seeded into the upper chamber and incubated for 36 h at

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37°C, 5% CO_2. Cells remained on the upper side of the filter membrane were gently removed with a cotton swab. Cells migrated or invaded to the bottom surface of the filter were quickly fixed with methanol for 10 min and stained with 1% Crystal Violet/20% methanol for 20 min. The stained cells from at least 8 random fields were examined under microscope at the magnification of 200×. Images were captured by cellSens software and processed by Adobe Photoshop without changing the original appearance.

Statistical Analysis

Immunoblot results were quantitated by using ImageJ software, and the optical density of each band was normalized to EGFR, ERK1/2, or β-actin. Statistical analyses were performed by using Graph Pad Prism 5 software. Data from three independent experiments were presented as means ± SEM. Statistical comparisons between groups were performed with paired *t*-test or one way ANOVA with *post hoc* test (Tukey). Data obtained from dose response experiments were analyzed by using non-linear curve fitting. Significance levels are indicated as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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Results

$GABA_BR$ agonist induced EGFR transactivation in human PCa cells

The expression of $GABA_BR$ was detected in human PCa cell lines PC-3 and LNCaP (**Fig. S1**). To identify whether activation of $GABA_BR$ hijacks RTKs signaling to modulate the fates of PCa cells, RTK array analysis was performed in PC-3 and LNCaP cells, which were stimulated with a specific $GABA_BR$ agonist, baclofen (**Fig. S2A**), for 5 min. The results showed that baclofen selectively upregulated the phosphorylation level of EGFR but not other RTKs in PC-3 cells (**Fig. 1A**).

To confirm the results of RTK array assay, PC-3 cell lysates prepared from untreated or baclofen treated cells were immunoprecipitated with pTyr antibody 4G10, and immunoblotted with EGFR antibody. As shown in **Figs. 1B and 1C**, baclofen increased the total phosphorylation level of EGFR for 2.063±0.627 (p<0.05, n=3) folds. Upon agonist stimulation, Y845 in the kinase domain and 8 tyrosine residues in the C-tail of EGFR (Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173) could be phosphorylated (Conte and Sigismund, 2016). Analysis of single site phosphorylation of EGFR revealed that baclofen significantly upregulated the levels of pY845, pY1045, pY1068, and pY1086 for 0.490±0.101 (p<0.05, n=3), 0.481±0.052 (p<0.01, n=3), 0.883±0.221 (p<0.05, n=3), and 1.070±0.356 (p<0.05, n=3) folds respectively, but showed no obvious influence on the phosphorylation of Y1148 and Y1173 (**Figs. 1B and 1C**). Phosphorylation of Y992 and Y998 was not detected in the presence of baclofen (data not shown), and phosphorylation of Y974 and Y1101 was not analyzed due to lack of antibodies.

$GABA_BR$ agonist induced activation of ERK1/2 in human PCa cells

GPCR-induced EGFR transactivation can activate ERK1/2 signaling cascades (Xiao et al., 2003; Zhou et al., 2012; George et al., 2013; Lai et al, 2016). To examine

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whether EGFR transactivation by $GABA_BR$ induced ERK1/2 activation, PC-3 cells were treated with baclofen or GABA for various period of time. The results showed that both baclofen and GABA induced a fast and transient phosphorylation of ERK1/2 in a time dependent manner, which peaked at 5 min, and returned to basal level after 60 min (**Figs. 2A and S3**). Baclofen also displayed dose-dependent effect on ERK1/2 activation, with the minimum concentration of 200 μM (**Fig. 2B**).

Pre-treatment of PC-3 cells with CGP54626 (**Fig. S2B**), a high affinity and highly selective $GABA_BR$ antagonist (Filip and Frankowska, 2008), abrogated baclofen induced ERK1/2 phosphorylation (**Fig. 2C**)**,** indicating that ERK1/2 activation was specifically mediated by $GABA_BR$.

Tarceva (also named erlotinib) and Iressa (also named gefitinib) are small molecule inhibitors of EGFR (Sharma et al., 2007). Pre-treatment of PC-3 cells with Tarceva or Iressa completely blocked baclofen-induced ERK1/2 phosphorylation (**Figs. 2E and 2F**), suggesting that baclofen induced ERK1/2 activation was dependent on EGFR transactivation by $GABA_BR$.

EGFR transactivation by $GABA_BR$ agonist was mediated by $G_{i/o}$ protein and ligand-dependent pathway

$GABA_BR$ is coupled to $G_{i/o}$ protein (Filip and Frankowska, 2008). Pre-treatment of cells with pertussis toxin (PTX), an inhibitor of $G_{i/o}$ protein, suppressed baclofen induced total and site-specific phosphorylation of EGFR and ERK1/2 phosphorylation (**Figs. 3A-3C**), implicating that baclofen induced EGFR transactivation and subsequent ERK1/2 activation was dependent on $G_{i/o}$ protein.

EGFR transactivation by GPCRs is mediated by either ligand-dependent or ligand-independent pathway. Ligand-dependent RTKs transactivation by GPCRs is dependent on the activation of MMPs (Cattaneo et al., 2014). EGFR ligands,

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including EGF, HB-EGF, AR, TGF-α, epiregulin, betacellulin, and epigen, are synthesized as transmembrane pro-ligands that are later processed by MMPs (Harris et al., 2003; Cattaneo et al., 2014). GM6001, a pan-MMPs inhibitor, abolished baclofen induced multisite phosphorylation of EGFR and ERK1/2 activation (**Figs. 3D-3F**), suggesting that baclofen induced EGFR transactivation was dependent on MMPs.

MMPs has been reported to mediate the shedding of pro-HB-EGF, -AR, or –TGF-α from cell surface and subsequent EGFR transactivation (Zhao et al., 2004; Kamanga-Sollo et al., 2014; Moody et al., 2014; Light and Hammes, 2015; Overland and Insel, 2015). EGFR neutralizing antibody, which blocked the ligand binding site of EGFR, abrogated baclofen-induced ERK1/2 phosphorylation in PC-3 cells (**Fig. 3G**). To identify which EGFR pro-ligand(s) was/were released by MMPs from cell surface, ELISA experiments were performed. As shown in **Fig. 3H**, the basal EGF level in control group was 3.177±0.292 pg/ml (n=3), baclofen treatment for 5, 10, or 15 min increased EGF level to 4.008±0.214 pg/ml ($p<0.05$, n=3), 4.888±0.361 pg/ml ($p<0.05$, n=3) and 6.056±0.235 pg/ml ($p<0.05$, n=3) respectively. Baclofen had no significant influence on HB-EGF level until fifteen-minute-treatment, while no upregulation of TGF-α and AR levels was detected in the presence of baclofen (Data not shown). These data indicated that production of EGF but not HB-EGF was upstream of ERK1/2 phosphorylation upon $GABA_BR$ activation, therefore, MMPs-mediated shedding of pro-EGF might be involved in baclofen induced EGFR signaling in PC-3 cells.

$GABA_BR$ agonist promoted migration and invasion of PC-3 cells

Baclofen has been shown to enhance the invasive ability of human prostate C4-2 cancer cells (Azuma et al., 2003). To analyze the effects of baclofen on migration and

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invasion of PC-3 cells, transwell experiments were performed. In the control group, the number of migrated cells was 105.8±9.176 per field of view (FV) (n=8), which increased to 167.6±7.573 (p<0.001, n=8), 204.5±5.292 (p<0.001, n=8), and 220.3±9.080 (p<0.001, n=8) in the presence of 50, 100, and 200 μM baclofen respectively (**Figs. 4A and 4B)**. $GABA_BR$ antagonist CGP54626 (5 μM) suppressed baclofen (100 μM) induced migration and invasion (**Figs. 4C-4D and 5A-5B**). Transwell experiments were then performed in the presence or absence of EGFR neutralizing antibody to explore the role of $GABA_BR$-induced ligand-dependent EGFR transactivation in migration and invasion. The results showed that EGFR neutralizing antibody remarkably suppressed baclofen induced migration and invasion **(Figs. 4E-4F and 5C-5D**), suggesting that ligand-dependent transactivation of EGFR by baclofen was essential for migration and invasion of PC-3 cells.

As a control, Baclofen displayed no effect on the survival of PC-3 cells after 96 h treatment. However, CGP54626 inhibited cell survival in a time- and dose-dependent manner, and 40 μM CGP54626 significantly inhibited cell survival after 48 h treatment (**Fig. S4**).

PAMs of $GABA_BR$ directly induced ERK1/2 activation as PAM agonists

CGP7930, rac-BHFF, and GS39783, the PAMs of $GABA_BR$ (**Fig. S2C**), are capable of modulating the efficacy of $GABA_BR$ induced signal transduction (Urwyler et al., 2001; 2003; 2005; Malherbe et al., 2008). PAM agonist activities of these three compounds have been reported previously (Binet et al., 2004; Tu et al., 2007; 2010; Malherbe et al., 2008; Gjoni and Urwyler, 2009; Koek et al., 2010; Gannon and Millan, 2011; Sun et al., 2016). To analyze whether these PAMs have intrinsic activity in cancer cells, PC-3 cells were incubated with PAMs without addition of agonist. As shown in **Figs. 6A and 6B**, CGP7930 can act as a PAM agonist to induce ERK1/2

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phosphorylation in a time and dose-dependent manner. Compared to baclofen induced transient activation of ERK1/2, CGP7930 induced a sustained phosphorylation of ERK1/2, which peaked at 10 min, and lasted for more than 2 h. 12 μM of CGP7930 was sufficient to activate ERK1/2, and 50 μM of CGP7930 resulted in maximum activation of ERK1/2 with an EC50 value of 20.56 μM. The profile of ERK1/2 phosphorylation induced by rac-BHFF was similar to that induced by CGP7930, which was sustained, peaking at 20 min, and lasting for more than 2 h. The minimum concentration of rac-BHFF to activate ERK1/2 was 75 μM (**Figs. 6D and 6E**). ERK1/2 activation induced GS39783 was similar to that induced by baclofen, which was transient, peaked at 5 min, and returned to basal level after 30 min. 0.6 μM of GS39783 was sufficient to induce ERK1/2 phosphorylation, and 5 μM GS39783 resulted in maximum phosphorylation of ERK1/2 with an EC50 value of 4.85 μM (**Figs. 6G and 6H**).

It has been documented that PC-3 cells may synthesize endogenous GABA (Ippolito and Piwnica-Worms, 2014). To clarify whether ERK1/2 activation induced by PAMs was due to a direct agonistic effect or the potentiation of endogenously produced GABA, PC-3 cells were pre-treated with $GABA_BR$ antagonist CGP54626 followed by PAMs. The results showed that CGP54626 did not abrogate ERK1/2 activation induced by CGP7930 and rac-BHFF (**Figs. 6C and 6F**), suggesting that CGP7930 and rac-BHFF had a direct agonistic effect on ERK1/2 activation. GS39783 induced activation of ERK1/2 was suppressed by CGP54626 (**Fig. 6I**), indicating that GS39783 may function as a pure PAM in PC-3 cells.

PAMs of $GABA_BR$ stimulated EGFR signaling in PC-3 cells

To investigate whether CGP7930, rac-BHFF, and GS39783 can activate EGFR signaling in PC-3 cells, immunoprecipitation experiments were performed. The results

Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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showed that these three PAMs significantly increased the total phosphorylation level of EGFR (**Figs. 7A-7C**). Further analysis showed that ERK1/2 phosphorylation induced by CGP7930, rac-BHFF, and GS39783 was suppressed by EGFR inhibitors Tarceva (**Figs. 7D-7F**), Iressa (**Figs. 7G-7H**), and EGFR neutralizing antibody (**Figs. 7J-7L**). These results suggested PAMs of $GABA_BR$ functioned similarly as baclofen to induce EGFR transactivation and EGFR ligand-dependent ERK1/2 activation.

CGP7930 promoted migration and invasion of PC-3 cells

Based on the time and dose effects of CGP7930 on cell survival (data not shown), transwell experiments were performed in the presence of maximal 4 μM CGP7930. As shown in **Figs. 8A and 8B,**. CGP7930 promoted migration of PC-3 cells in a dose-dependent manner, and 1 μM CGP7930 was sufficient to enhance cell migration. Transwell experiments were also performed in the presence or absence of EGFR neutralizing antibody to explore the role of CGP7930 induced EGFR transactivation in migration and invasion. The results showed that EGFR neutralizing antibody blocked CGP7930 induced migration (**Figs. 8C and 8D)** and invasion (**Figs. 8E and 8F)**, suggesting that ligand-dependent transactivation of EGFR by CGP7930 was important for migration and invasion of PC-3 cells.

Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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Discussion

EGFR family has received much attention in pharmacological research due to their strong association with malignant proliferation (Gallick et al., 2012). In addition to direct activation by specific ligands, EGFR family members could also be transactivated by a variety of GPCR agonists (Daub et al., 1996; Zhou et al., 2012; Cattaneo et al., 2014; Muñoz-Moreno et al., 2014; Lai et al., 2016; Wang, 2016). In this study, we demonstrated for the first time that specific activation of $GABA_BR$, a neurotransmitter receptor belonging to GPCR family, selectively induced EGFR transactivation in PC-3 cells. Published data revealed that EGFR ligands can induce multisite phosphorylation of EGFR at Y845, Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173 (Conte and Sigismund, 2016). Single site phosphorylation of EGFR at Y845 or Y992 has been detected in PCa cells treated with neuropeptides (GPCR agonists) (Amorino et al., 2007; DaSilva et al., 2013). Dual site phosphorylation of EGFR at Y845 and Y1068 was observed in rat-1 cells stimulated with bombesin and lysophosphatidic acid (GPCR agonists) (Santiskulvong et al., 2003). We identified that baclofen selectively induced the multisite phosphorylation of EGFR at Y845, Y1045, Y1068, and Y1086. Phospho-Y1045, -Y1068, and -Y1086 in EGFR are involved directly or indirectly in the recruitment of Cbl to EGFR, leading to ubiquitination of EGFR. Extensively ubiquitinated EGFR might be internalized through non-clathrin endocytosis (NCE) and degraded in lysosomes (Conte and Sigismund, 2016). It is of particular interest that baclofen selectively induced multisite phosphorylation of tyrosine residues that are involved in EGFR ubiquitination. It is worth to know whether baclofen induces massive ubiquitination and NCE biased internalization of EGFR (compared to classical clathrin-mediated endocytosis) in PC-3 cells.

EGFR transactivation by GPCRs occurs mainly through ligand-dependent and ligand-independent mechanisms. In the ligand-dependent mechanism, MMPs mediate the shedding of pro-ligands from cell surface and subsequent RTK transactivation by GPCRs (Cattaneo et al., 2014). It has been reported that MMPs mediated the shedding of pro-HB-EGF and subsequent EGFR transactivation in PC-3 cells treated with neurotensin, bombesin, tetradecanoyl-phorbol-13-acetate, or calcium-sensing receptor stimulus (increased level of extracellular Ca^{2+}) (Prenzel et al., 1999; Madarame et al., 2003; Hassan et al., 2004; Yano et al., 2004). Our study suggested that MMPs may promote the cleavage of pro-EGF from cell surface and EGFR transactivation in PC-3 cells stimulated with baclofen. A significant increase of HB-EGF level was detected 10 min later than that of pro-EGF. Therefore, EGF might be the main ligand that induces the fast transactivation of EGFR. AR and TGF-α was not detected in this study, probably due to lack of expression or shedding of these two ligands, or the concentration of these two ligands were too low to be detected.

We have reported previously that $GABA_BR$ induced transactivation of IGF-1R in neurons in a ligand-independent pathway, which was mediated by phospholipase C, cytosolic Ca^{2+}, and focal adhesion kinase 1 (Tu et al., 2010), while this study demonstrated that $GABA_BR$ induced EGFR transactivation in a ligand-dependent pathway, indicating that a single GPCR may transactivate two RTKs by different mechanisms, which might be tissue specific. So far, most studies suggested one GPCR to one RTK transactivation pathway (Cattaneo et al., 2014; Wang, 2016). However, similar to our results, several other studies suggested a single GPCR to two RTKs pathway. In PC-3 and LNCaP cells, neuropeptides induced Src- and MMP-dependent EGFR transactivation (pY992) and subsequent IGF-1R phosphorylation (DaSilva et al., 2013). In PC-3 cells, growth hormone-related

Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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hormone stimulated a rapid (30 s) ligand-independent activation of EGFR and HER2, and a slow (30 min) ligand-dependent activation of EGFR and HER2 (Muñoz-Moreno et al., 2014). In primary cultures of Müller cells of the guinea pig, P2Y receptor agonist ATP induced transactivation of PDGFR and EGFR in a ligand-dependent pathway (Milenkovic et al., 2003). Therefore, GPCR agonist(s) may induce a more global RTK response depending on cellular context.

ERK1/2 signaling cascade, one of the overlapping signaling pathways downstream of GPCRs and RTKs, is involved in the development and progression of various human cancers (Osborne et al., 2012). This study revealed that both agonist and PAMs of $GABA_BR$ can induce ERK1/2 activation in an EGFR ligand-dependent pathway. Interestingly, CGP7930 and rac-BHFF induced ERK1/2 activation was not antagonized by $GABA_BR$ antagonist CGP54626, suggesting a direct agonist effect of these two compounds, which is consistent with data published by us and other research groups (Binet et al., 2004; Tu et al., 2007; 2010; Malherbe et al., 2008; Koek et al., 2010; Sun et al., 2016). ERK1/2 activation induced by GS39783 was blocked by CGP54626, indicating that GS39783 may function as a pure PAM in PC-3 cells. But we didn't exclude the possibility that GS39783 may have a direct agonist activity. As described in Materials and Methods, serum starved PC-3 cells were washed with SFM before PAMs treatment, so the endogenous level of GABA in PC-3 cells might be too low to be effective. The different effects of CGP54626 on ERK1/2 activation induced by CGP7930, rac-BHFF, and GS39783 suggested that $GABA_BR$ PAMs may have distinct binding sites on the transmembrane domain of $GABA_{B2}$ subunit. Upon CGP54626 binding, both $GABA_{B1}$ and $GABA_{B2}$ subunits undergo a conformational change, which may distort the binding site for GS39783 but retain the binding capacity for CGP7930 and rac-BHFF.

It has been reported that GABA simulated proliferation of PC-3 cells (Wu et al., 2014). In this study, baclofen displayed no effect on the survival of PC-3 cells, but $GABA_BR$ antagonist CGP54626 inhibited cell survival. Therefore, the effect of baclofen on the survival of PC-3 cells was probably masked by endogenous GABA; when the effect of GABA was blocked by $GABA_BR$ antagonist, cell survival was suppressed.

This study showed that both baclofen and CGP7930 significantly promoted migration and invasion of PC-3 cells, but EGFR neutralizing antibody abolished such effects, indicating that $GABA_BR$ induced EGFR transactivation was essential for enhanced migration and invasion of PC-3 cells. Similar results were obtained in prostate cancer cells C4-2, renal cell carcinoma cells, and mouse 4T1 cells treated with GABA or baclofen (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014).

Taken together, our study revealed that $GABA_BR$ agonist selectively induced multisite phosphorylation of EGFR at Y845, Y1045, Y1068 and Y1086, which was dependent on $G_{i/o}$ protein and MMPs mediated cleavage of pro-EGF, resulting in ERK1/2 activation. PAMs of $GABA_BR$ (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Baclofen and CGP7930 induced EGFR transactivation played an important role in migration and invasion of PC-3 cell (Fig. 9). To our knowledge, this is the first time to identify the mechanism of $GABA_BR$ induced EGFR signaling in cancer cells. Since clinical trials of EGFR inhibitors in patients with castration-resistant PCa produced disappointing results (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014), neurotransmitter GABA and its receptor $GABA_BR$ might be potential targets in the clinical treatment of PCa patients.

Acknowledgements

We thank Drs. J-P. Pin and P. Rondard from Institut de Génomique Fonctionnelle, Université de Montpellier, Montpellier, France for their advice on this study.

Authorship contributions

Participated in research design: XJ, JL

Conducted experiments: SX, XJ, CH, YZ, SW, HL, ZZ

Performed data analysis: SX, XJ, CH, YZ, SW, HL

Wrote or contributed to the writing of the manuscript: XJ, JL, SX, CH

Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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Footnotes

- Financial support: The study was financially supported by grants from National Natural Science Foundation of China [31170790, 31225011, 31420103909, 31371423]; start funding from The Ministry of Education, China, independent innovation research funding from Huazhong University of Science and Technology, Wuhan, China [M2009047]; the Fundamental Research Funds for the Central Universities, China [0118170132/2016YXMS25]; the Natural Science Foundation of Hubei province [2014CFA010]; the Program of Introducing Talents of Discipline to Universities from the Ministry of Education of China [B08029], and Program for Changjiang Scholars and Innovative Research Team in University [PCSIRT: IRT13016].

- SX and CH contributed equally to this work.

Molecular Pharmacology Fast Forward. Published on April 19, 2017 as DOI: 10.1124/mol.116.107854
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Figure Legends

Fig. 1. $GABA_BR$ induced transactivation of EGFR in PC-3 cells. PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h and incubated with or without 200 μM baclofen at 37°C for 5 min. (A) 300 μg of TCL were subjected to RTK array assay as described in Materials and Methods. (B) 500 μg of TCL were immunoprecipitated with 4G10-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), and β-actin were assessed by immunoblot analysis (B) and quantitated by ImageJ (C). Data were normalized to total EGFR, expressed as the fold change (means ± SEM; n=3), and analyzed by paired *t*-test. * $p<0.05$, ** $p<0.01$ (*v.s.* control).

Fig. 2. Baclofen induced ERK1/2 activation in a time- and dose-dependent manner. PC-3 cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h, replaced with SFM and incubated at 37°C for 30 min. (A) Cells were treated with 200 μM baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (B) Cells were incubated with 0, 50, 100, 150, 200, 250, or 300 μM baclofen at 37°C for 5 min. (C) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 200 μM baclofen at 37°C for 5 min. (D, E) Cells were incubated with 5 μM Taceva (D) or 5 μM Iressa (E) at 37°C for 8 h and then treated with 200 μM baclofen at 37°C for 5 min. Cells treated with 0.05% DMSO were used as controls. At the end of incubation, equal amounts of TCLs were subjected to immunoblot analysis. The levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (means ± SEM; n=3). Data were analyzed using non-linear curve fitting (B) or one way ANOVA with *post hoc* test (Tukey) (C-E). *** $p<0.001$. ns: not significant.

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Fig. 3. EGFR transactivation induced by baclofen was dependent on $G_{i/o}$ protein and MMPs. (A) PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h in the presence or absence of 200 ng/ml PTX. Cells were then incubated with or without 200 μM baclofen at 37°C for 5 min. 500 μg of TCL were immunoprecipitated with EGFR-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β-actin were assessed by immunoblot analysis (A) and quantitated (B). (C) The levels of pERK1/2, ERK1/2, and β-actin in samples prepared in (A) were analyzed by immunoblot assay. (D) Serum starved cells grown in 3.5 cm plates were pre-incubated with 20 μM GM6001 (GM) or 0.05% DMSO at 37°C for 1 h, and then treated with 200 μM baclofen at 37°C for 5 min. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β-actin were assessed by immunoblot analysis (D) and quantitated (E). (F) The levels of pERK1/2, ERK1/2, and β-actin in samples prepared in (D) were analyzed by immunoblot assay. (G) Serum starved cells grown in 3.5 cm plates were pre-incubated with or without EGFR neutralizing antibody (2 μg/ml) at 37°C for 1 h, and then treated with 200 μM baclofen at 37°C for 5 min. Equal amounts of TCLs were subjected to immunoblot analysis to assess the levels of pERK1/2, ERK1/2, and β-actin. (H) Serum starved cells grown in 3.5 cm plates were treated with 200 μM baclofen at 37°C for 0, 5, 10, or 15 min, CM were then collected and the levels of EGF and HB-EGF were analyzed by ELISA, which were performed in triplicates and repeated three times. Data were expressed as the fold change (means ± SEM; n=3), and analyzed by one way ANOVA with *post hoc* test (Tukey) (B-C, E-G) or paired *t*-test (H). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ns: not significant.

Fig. 4. Baclofen promoted migration of PC-3 cells. 700 μl of medium containing 10%

FBS was added in the lower chamber of the transwell. PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 5×10^4 cells/200 μl in the presence of 0, 50, 100, or 200 μM baclofen were added into the upper chamber and incubated for 36 h at 37°C, 5% CO_2. (C) 5×10^4 cells/200 μl in the presence or absence of 100 μM baclofen (Bac) or 5 μM CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. (E) 5×10^4 cells/200 μl in the presence or absence of 100 μM baclofen or EGFR neutralizing antibody (Ab) (0.5 μg/ml) were added to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. At the end of incubation, cells remained on the upper side were fixed, stained, and observed under microscope. Images from at least 8 random fields were captured by cellSens. The numbers of migrated cells in panels A, C, and E were counted, and expressed as means ± SEM (n=8) in panels B, D, and F respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel. Data were analyzed by paired *t*-test (B) or one way ANOVA with *post hoc* test (Tukey) (D, F). *** $p<0.001$. Scale bar: 200 μm.

Fig. 5. Baclofen promoted invasion of PC-3 cells. 700 μl of medium supplemented 10% FBS was added in the lower chamber, and the filter of the transwell was coated with 70 μl of Matrigel (1 mg/ml). PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 1×10^5 cells/200 μl in the presence or absence of 100 μM baclofen or 5 μM CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. (C) 1×10^5 cells/200 μl in the presence or absence of 100 μM baclofen or EGFR neutralizing antibody (Ab) (0.5 μg/ml) were added to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. Cells remained on the upper side of the filters were fixed, stained, and observed under microscope. Images from at least 8 random fields were captured by cellSens. The

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numbers of invasive cells in panels A and C were counted and expressed as means ± SEM (n=8) in panels B and D respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel, and statistical analysis was performed using one way ANOVA with *post hoc* test (Tukey).*** $p<0.001$. Scale bar: 200 μm.

Fig. 6. $GABA_BR$ PAMs induced ERK1/2 activation in PC-3 cells. Cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h, replaced with SFM and incubated at 37°C for 30 min. (A) Cells were treated with 50 μM CGP7930 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (B) Cells were incubated with 0, 3, 6, 12, 25, 50, or 100 μM CGP7930 at 37°C for 10 min. (C) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 50 μM CGP7930 37°C for 10 min. (D) Cells were treated with 100 μM rac-BHFF at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (E) Cells were incubated with 0, 25, 50, 75, 100, 125, or 125 μM rac-BHFF at 37°C for 20 min. (F) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 100 μM rac-BHFF at 37°C for 20 min. (G) Cells were incubated with 10 μM GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (H) Cells were treated with 0, 0.6, 1.2, 2.5, 5, 10, or 20 μM GS39783 at 37°C for 5 min. (I) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 10 μM GS39783 at 37°C for 5 min. At the end of incubation, equal amounts of TCLs were resolved by SDS-PAGE followed by immunoblot analysis; the levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (means ± SEM; n=3). Statistical analysis was performed using non-linear curve fitting (B, E, H) or one way ANOVA with *post hoc* test (Tukey) (C, F, I). ** $p<0.01$, *** $p<0.001$. ns: not significant.

Fig. 7. $GABA_BR$ PAMs induced EGFR signaling in PC-3 cells. (A-C) PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h and incubated with or without 50 μM CGP7930 (A), 100 μM rac-BHFF (B), or 10 μM GS39783 (C) at 37°C for 10, 20, or 5 min, respectively. 500 μg of TCL were immunoprecipitated with 4G10-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pERK1/2, ERK1/2, and β-actin were analyzed by immunoblot assay. (D-L) Cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h and pre-treated with 5 μM Taceva (D-F), 5 μM Iressa (G-I), or 2 μg/ml EGFR neutralizing antibody (J-L) at 37°C for 8, 8, or 1 h, respectively. Cells were then incubated with 50 μM CGP7930 (D, G, J), 100 μM rac-BHFF (E, H, K), or 10 μM GS39783 (F, I, L) at 37°C for 10, 20, or 5 min, respectively. The levels of pERK1/2, ERK1/2, and β-actin were analyzed by immunoblot assay and quantitated. Data were normalized to total EGFR or ERK1/2, expressed as the fold change (means ± SEM, n=3), and analyzed by paired *t*-test (A-C) or one way ANOVA with *post hoc* test (Tukey) (D-L). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ns: not significant.

Fig. 8. CGP7930 promoted migration and invasion of PC-3 cells. 700 μl of medium containing 10% FBS was added in the lower chamber of the transwell. PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 5×10^4 cells/200 μl in the presence of 0, 1, 2, or 4 μM CGP7930 were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. (C) 5×10^4 cells/200 μl in the presence or absence of 4 μM CGP7930 or 0.5 μg/ml EGFR neutralizing antibody were added to the upper chamber and incubated for 36 h at 37°C, 5% CO_2. (E) The filter of the transwell was coated with 70 μl of Matrigel (1 mg/ml). 1×10^5 cells/200 μl in the presence or absence of 4 μM CGP7930 or 0.5 μg/ml EGFR neutralizing antibody were added to the upper chamber and incubated for 36 h at 37°C, 5% CO_2.

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Images of cell migration from at least 8 random fields were captured by cellSens. The numbers of migrated or invaded cells in panels A, C, and E were counted and expressed as means ± SEM (n=8) in panels B, D, and F respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel. Statistical analysis was performed using paired *t*-test (B) or one way ANOVA with *post hoc* test (Tukey) (D, F). ** $p<0.01$, *** $p<0.001$. ns: not significant. Scale bar: 200 μm.

Fig. 9. Diagram of $GABA_BR$ induced EGFR transactivation in PC-3 cells. Upon agonist binding, $GABA_BR$ activates $G_{i/o}$ protein, and MMPs mediated cleavage of pro-EGF from cell surface leads to multisite phosphorylation of EGFR at Y845, Y1045, Y1068, Y1086, and subsequent ERK1/2 activation. In addition to baclofen, PAMs of $GABA_BR$ (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and increase ERK1/2 activity. Both baclofen and CGP7930 promote cell migration and invasion in an EGFR transactivation dependent pathway.

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Fig. 1

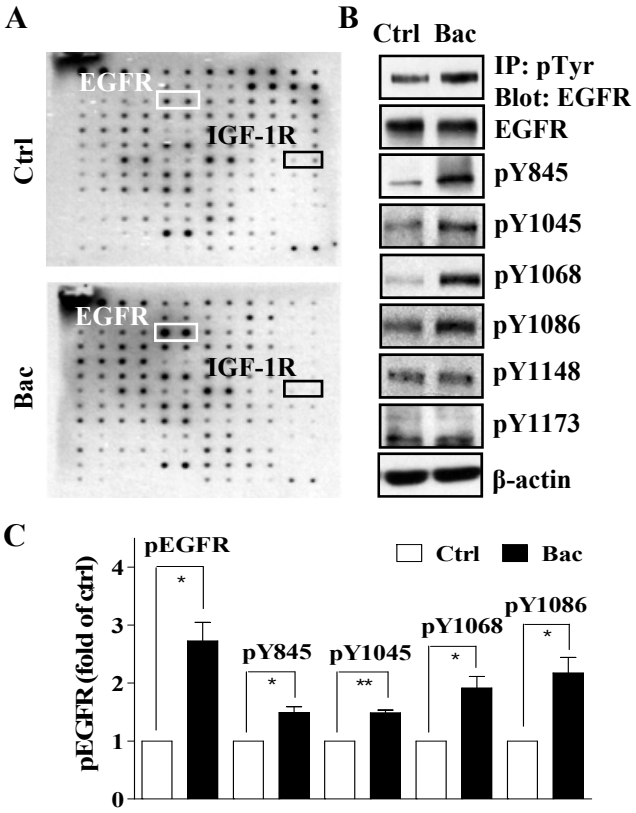

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Fig. 2

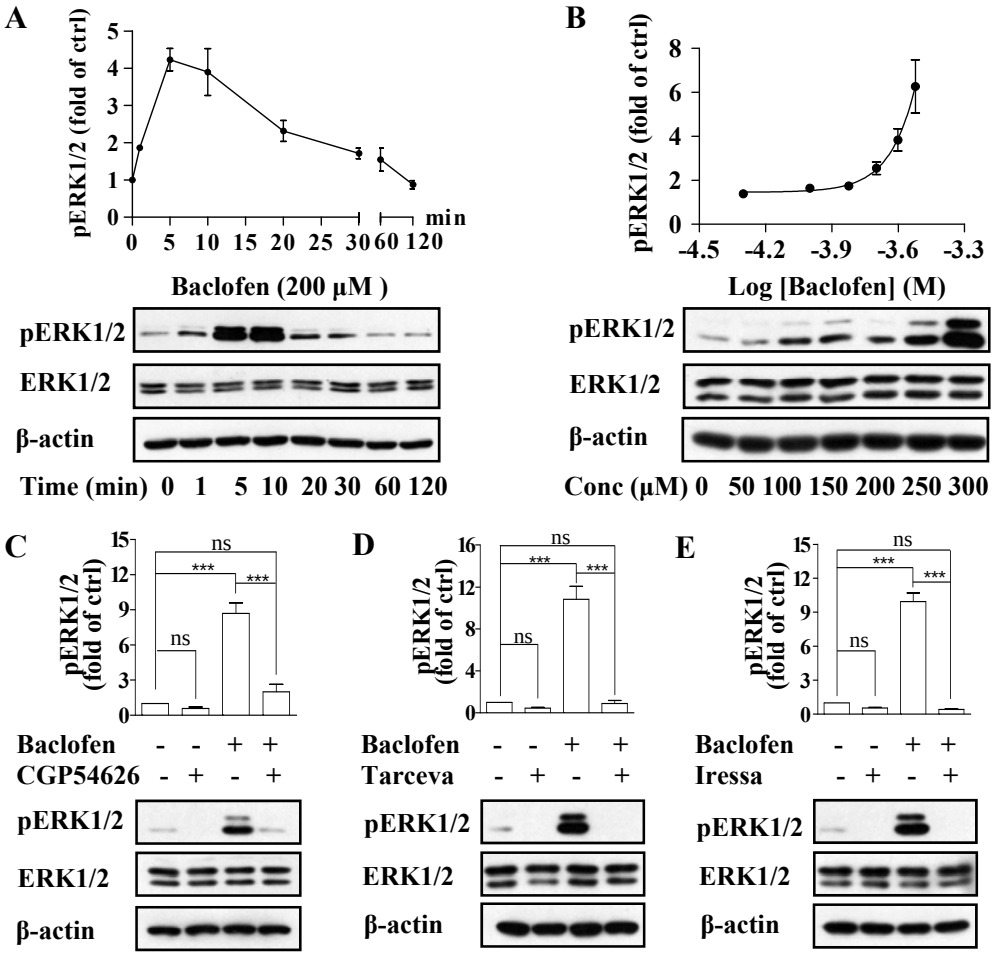

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Fig. 3

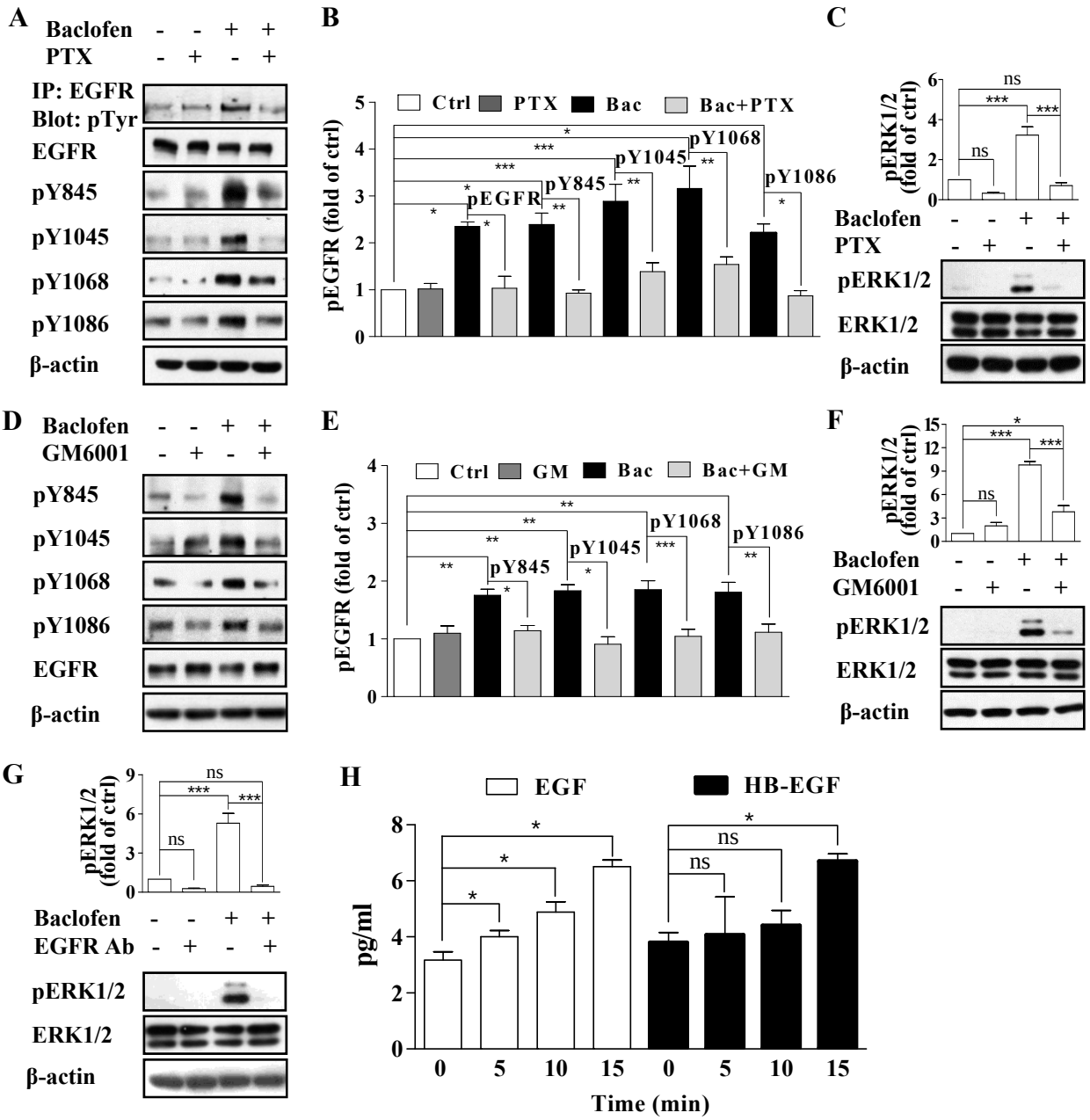

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Fig. 4

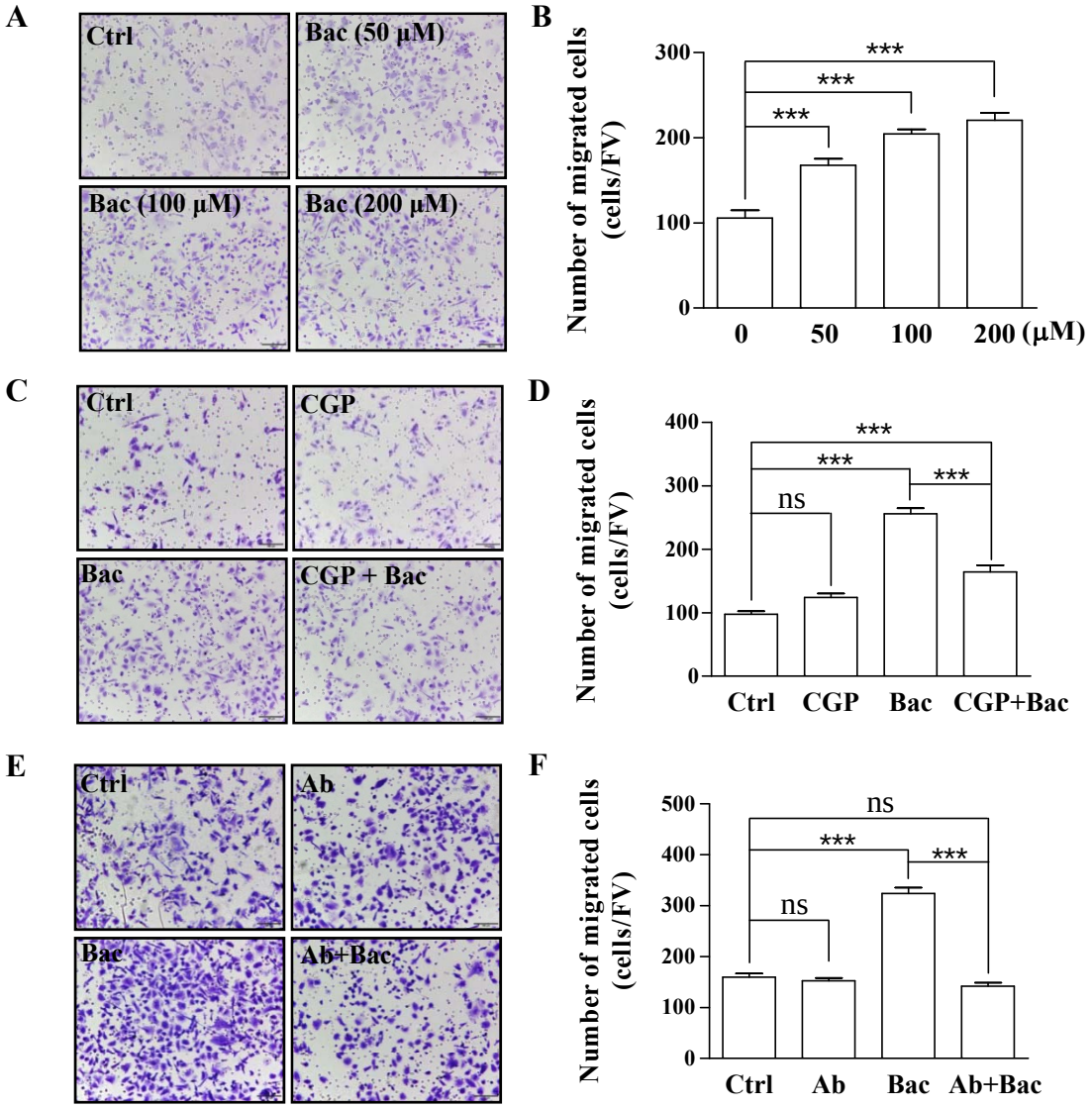

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Fig. 5

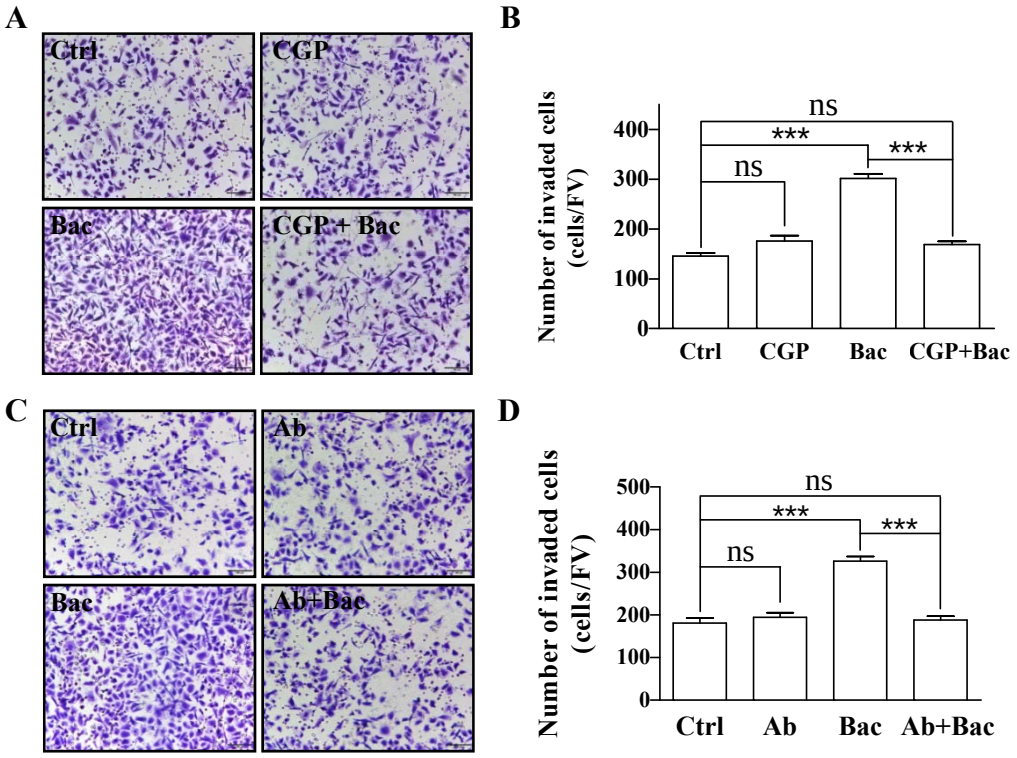

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Fig. 6

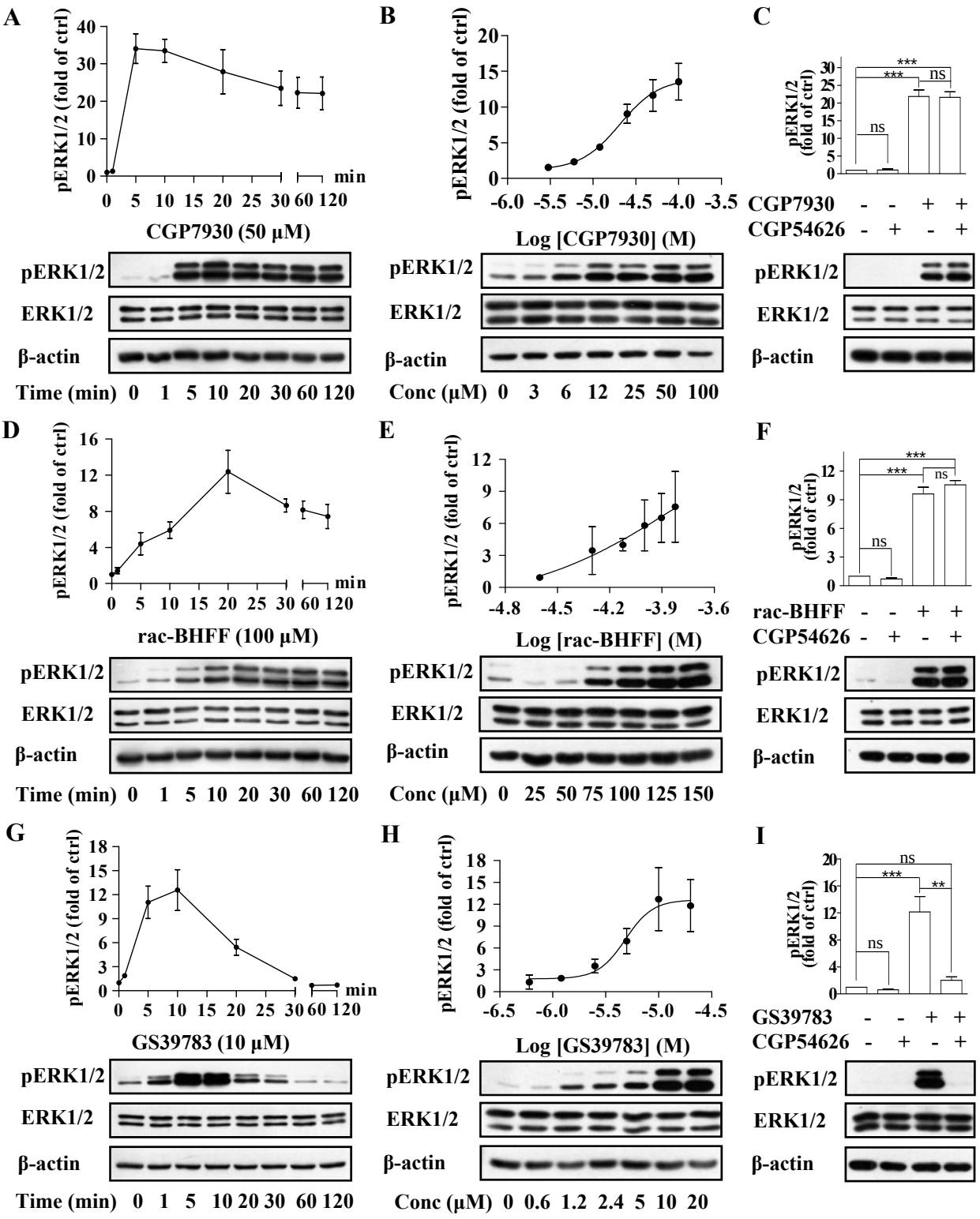

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Fig. 7

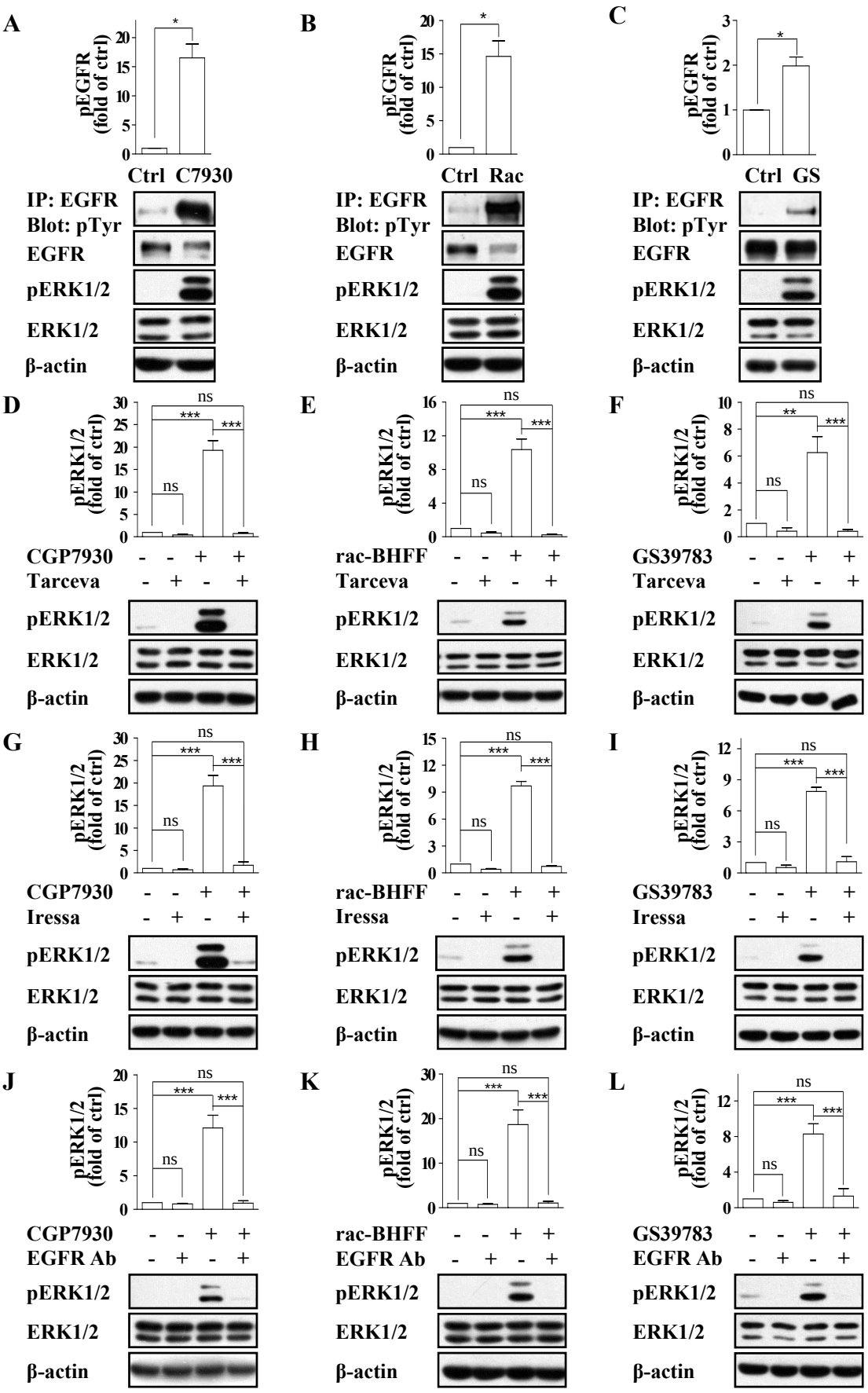

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Fig. 8

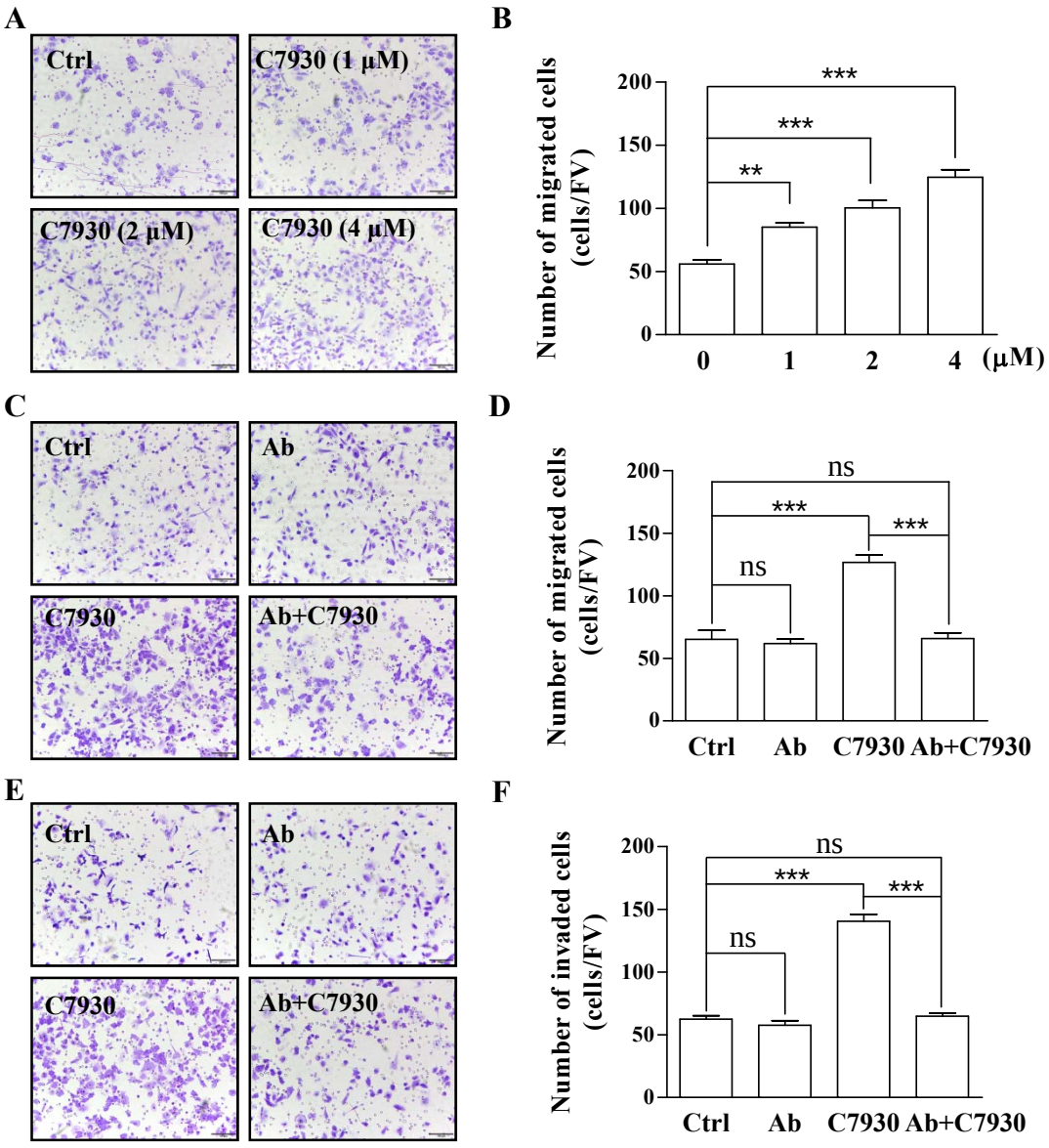

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Fig. 9