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**Neuropeptide Y receptors mediate ERK1/2 activation via transactivation of the
IGF receptor**

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Abstract

Neuropeptide Y binds to G-protein coupled receptors whose action results in inhibition of adenylyl cyclase activity. Using HEK293 cells stably expressing the native Y₁ neuropeptide Y receptors, we find that the NPY agonist elicits a transient phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2). We first show that ERK1/2 activation by the Y₁ receptor is dependent on heterotrimeric G_{i/o} since it is completely inhibited by pre-treatment with pertussis toxin and it is internalization-independent since mutated Y₁ receptor which do not recruit β -arrestins, exerted ERK1/2 activation to the same extent. We next show that this classical MAPkinase pathway activation, since inhibited by the MEK inhibitor U0126, is independent on calcium signaling at the Y₁ receptor (no effect upon inhibition of phospholipase C, protein kinase C or protein kinase D) but dependent on a G α_q -mediated signalling pathway activating PI3-kinase. Although the Epidermal-Growth factor tyrosine receptor kinase inhibition did not influence NPY-induced ERK 1/2 activation, we show that the inhibition of Insulin Growth factor receptor IGFR by AG1024 completely block ERK1/2 activation by Y₁ receptor. This G α_q -PI3K-AG1024-sensitive pathway does not involve activation of IGFR through the release of a soluble ligand by metalloproteinases since it is not inhibited by marimastat. In addition, we found that a similar pathway, sensitive to wortmannin-AG1024 but insensitive to marimastat, was also found to be implicated in activation of ERK1/2 in HEK293 cells by endogenously expressed GPCRs coupled to Gq-protein (muscarinic M3 receptors) or coupled to Gs-protein (endothelin ETB receptors).

Keywords: neuropeptide Y; G protein-coupled receptor; extracellular-regulated protein kinase; human embryonic kidney, IGFR, beta-arrestin, marimastat, AG1024

Introduction

Neuropeptide Y (NPY) is a widely distributed peptide in the central and peripheral nervous system (Berglund et al., 2003). The physiological functions of this neurohormone are mediated by a family of five receptors which belong to the class A of the G-protein coupled receptors (GPCRs) and which are expressed in neuronal and non-neuronal tissues. Classically, signalling of GPCRs is mediated by receptor coupling to heterotrimeric G proteins that activate a variety of downstream cellular effectors. The signalling pathway of the NPY family of receptors is known to be coupled to the $G_{\alpha i}$ -protein, which leads to the inhibition of the adenylyl cyclase and inhibition of cAMP accumulation (Michel et al. 1998).

The classical paradigm of signalling via inhibition of adenylyl cyclase does not always adequately explain the full range of the effects of the activation of the NPY family receptors. Recent studies have shown that activation of the NPY receptors lead to cell proliferation, neurogenesis or gene transcription, via the phosphorylation of the extracellular signal regulated protein kinases 1 and 2 (Howell et al., 2005; Thiriet et al., 2011; Shimata et al., 2012). GPCRs can mediate ERK 1/2 activation by different mechanism, being able to induce activation of both G protein-dependent and G protein-independent signalling pathways.

Hence, growth promoting effects of GPCR stimulation have been shown to be mediated through transactivation of growth factor receptor tyrosine kinases (RTKs of epidermal growth factor (EGF), insulin growth factor (IGF), platelet-derived growth factor PDGF, fibroblast growth factor FGF, vascular endothelial growth factor VEGF and TrkA receptors, Wetzker and Böhmer 2003). Different mechanisms of transactivation have been described. It includes the extracellular release of RTK ligand by secretion or by shedding of a ligand through the processing of pre-ligand by plasma membrane metalloproteinases. Transactivation can also occurs inside the cell for example, activation of the c-Src-kinase is a general mechanism described, alternatively, it has been proposed to occur via direct molecular interaction between RTK and GPCRs or via inactivation of protein-tyrosine phosphatases that repress RTK (Prenzel et al. 1999, Pierce et al. 2001, Rozengurt 2007, Wetzker and Böhmer 2003, Oligny-Longpré et al. 2012). However, transactivation is not an essential

requirement in the activation of the ERK1/2 signalling cascade induced by GPCR activation (Neve, 2005, Shah 2006).

During the past decade, evidences have emerged showing that a variety of GPCRs can also couple to other adaptor proteins independently of heterotrimeric G-protein (reviewed by DeFea, 2008; Shenoy and Lefkowitz, 2011). Among these transducers are the scaffolding proteins β -arrestin-1 and β -arrestin-2 which are cytosolic endocytic adaptors playing an important role in GPCRs desensitization, internalization and trafficking. Upon GPCR activation, they can also function as molecular mediators by acting especially on ERK1/2 signal transduction pathway (Shenoy et al., 2006). It is increasingly evident that β -arrestins can bind to MAP kinases and facilitate their activation (DeFea for review 2008?).

In previous studies, we have shown that, unlike NPY Y_2 receptors, NPY Y_1 receptors rapidly internalize through clathrin-coated pits and recycle (Gicquiaux et al. 2002, Ouedraogo *et al.*, 2008). Upon stimulation by NPY, the endocytic mechanism involves cytosolic β -arrestins activation and translocation to the plasma membrane. Deletion of the carboxylterminal tail of the receptor ($Y_1 \Delta 42$ receptor) or substitution of key putative phosphorylated residues by alanine in the C-terminal tail of the receptor (Y_1 TDST-A receptor) abolish both internalization and β -arrestin activation. Likewise, in the human NPY Y_2 receptor which does not internalize, no significant activation of β -arrestin activation was detected (Ouedraogo et al. 2008).

Therefore, the present study was undertaken in order to better understand the involvement of G-protein- and/or β -arrestins-mediated signalling in the ERK1/2 phosphorylation pathway in NPY receptor activation. Different aspects of NPY-induced ERK1/2 activation were evaluated by the use of the mutant receptors that do not recruit beta-arrestins and of specific inhibitors in order to sequentially investigate the downstream signalling mechanisms upon activation of the receptor.

Material and methods

Cell culture, cDNA and treatments

Control and transfected HEK293 cells were cultured to ~ 80% confluence in T-75 flasks in MEM with Earle's salt supplemented with 10 % fetal calf serum, 2mM glutamine and 1% antibiotics (penicillin/streptomycin) (Gicquiaux et al., 2002). For the specific experiments, transfected HEK293 cells with the respective receptors were seeded in 6-well plates (200 000 - 300 000 cells/well) in order to attain 70-80% confluence after 2 days of culture. Cells were then serum starved overnight (roughly 16H) before activation with NPY with or without different inhibitors for different times as described in the individual experiments. The stimulation was terminated by aspirating the culture medium on ice and lysing the cells in 250 μ L of cold RIPA buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with EDTA-free anti-protease and anti-phosphatase cocktails.

cDNA of the human NPY Y₁ receptor was kindly provided by Prof. H. Herzog (Garvan Institute, Sydney, Australia). The cloning schemes for the different mutants of the native Y₁ receptor used in the present study were previously described (Ouedraogo et al., 2007; Lecat et al., 2011). All constructions were verified by sequencing and stably transfected cells were established by selective antibiotic selection.

SDS page and western blot analysis

Lysates were sonicated three times on ice using a Branson Sonifier at constant power, output = 2.5, and continuous sonication for 1 second. After sonication, insoluble elements were cleared by centrifugation at maximum speed (~14k) for 5 minutes at 4°C. Proteins concentration was determined using Bradford reagent. Homogenates of cell lysate were mixed with 4x laemmli buffer, denatured 5 min at 95°C, loaded on a 12% SDS-PAGE gel (20 μ g/lane). Protein samples were transferred to immobilon PVDF membranes. Membranes were blocked 1h (using 5% nonfat dried milk in PBS, pH 7.2, tween 0.1%), probed overnight with rabbit (polyclonal) anti-ERK1/2 [pTpY185/187] phosphospecific antibody (1:5000,

Invitrogeb, Camarillo, CA). The strips were thoroughly washed with PBS, Tween 0.1% then incubated 40 min with secondary antibody horseradish peroxidase-conjugated donkey anti rabbit diluted at 1/10 000. The immunoreactives bands were visualized using Immobilon western chemoluminescent HRP substrate detection reagents (Merck-Millipore) either with hyperfilm or LAS 4000 camera, bands were quantified using respectively either ImageJ or ImageQuant softwares (GE-Healthcare). The data were normalized to the maximal phospho-ERK1/2 response for each set of experiment.

Antibodies desorbing and second antibodies incubation

Antibodies were stripped from the membrane strips by 3 incubations of 15 min in a desorbing solution (200 mM HCl, 3% glycine). Membranes were washed thoroughly with PBS, Tween 0.1%), then blocked 1h using 5% nonfat dried milk in PBS, pH 7.2, tween 0.1%. Membrane strips were incubated overnight at 4°C with anti-ERK1/2pAb antibody (1:10 000, Enzo Life Sciences, France). The strips were thoroughly washed with PBS, Tween 0.1% then incubated 40 min with secondary antibody horseradish peroxidase-conjugated donkey anti rabbit diluted at 1/10000. For each immunoblot, we used actin as loading control. Mouse monoclonal anti-actin clone C4 1/100 000 was from Merck-Millipore, HRP-goat anti-mouse from GE-Healthcare.

Peptides and chemicals

Human NPY was from Neosystem (France). PP2 and wortmannin were from Enzo Life Science (Villeurbanne, France). AG1478, AG1024, Gö6983, gallein and marimastat were from Tocris Bioscience (Bristol, UK). U73122, U0126, pertussis toxin (PTX) were from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France).

Results

NPY Y₁ receptors mediate ERK1/2-phosphorylation via a Gi-dependent pathway.

To determine the temporal pattern of ERK1/2 activation mediated by NPY, we examined the kinetic of ERK1/2 phosphorylation following stimulation by NPY in Y₁ receptors expressing HEK293 cells. For this time-course study cells were incubated with 100 nM NPY for several time-points. Kinetic analysis by western-blot revealed that NPY treatment induced a rapid phosphorylation of ERK1/2 with a maximal activity at 5 min (Fig. 1A). The return to almost basal levels of phosphorylation was achieved by 20 min. NPY induced a concentration-dependent increase in levels of ERK1/2 phosphorylation in cells expressing the human Y₁ receptor (Fig. 1B) with an EC₅₀ of 0.3 nM. In order to assess the role of Gi/o proteins in NPY mediated ERK1/2 phosphorylation, the effect of selective inhibition of Gi-dependent signalling in human Y₁ receptor expressing cell lines was examined. PTX pre-treatment, concentration-dependently, inhibited ERK1/2 phosphorylation induced by 100 nM NPY indicating use of Gi/o proteins in the ERK signalling pathway (Fig. 1C). The inhibitor of the mitogen-activated protein (MAP) kinase/ERK kinase (MEK), U0126, abolished in a concentration dependent manner, ERK1/2 phosphorylation in response to 300 pM NPY indicating a conventional mechanism of ERK1/2 activation in the NPY Y₁ receptor (Fig. 1D).

NPY Y₁ and Y₂ receptors mediate ERK1/2-phosphorylation via an internalization-independent pathway.

To test the role of beta-arrestins in MAPkinase signaling at the level of activated NPY receptors, we have analysed the signaling induced by receptors that do not recruit beta-arrestins such as NPY Y₂ receptors and mutants of Y₁ receptors (Ouedraogo et al. 2008).

Kinetic analysis by western-blot revealed that NPY treatment induced a rapid phosphorylation of ERK1/2 by Y₂ receptors with a lower intensity as compared to phosphorylation of ERK1/2 by wild-type Y₁ receptors (Fig. 2A). It also peaked with a maximal activity at 10 min instead of 5 min (Fig. 2B). Exposure of cells expressing the human Y₂ receptor resulted in a concentration-dependent increase in ERK1/2 phosphorylation with an EC₅₀ value of 10 nM (Fig. 2C). PTX pre-treatment,

concentration-dependently inhibited ERK1/2 phosphorylation induced by 100 nM NPY indicating use of Gi/o proteins in the ERK signalling pathway (Fig. 2D).

Similarly, kinetic analysis by western-blot revealed that NPY treatment induced a rapid phosphorylation of ERK1/2 with a maximal activity at 5 min for cells expressing the human Y₁ Δ42 and Y₁ TDST-A receptors (Fig3A and 3B respectively). Thus, Y₁ mutants receptors that do not recruit beta-arrestins and do not internalized trigger MAPkinase activation to the same extent as wild-type receptors ruling out a mechanism of activation mediated by platforms of beta-arrestins bound to Y₁ receptors in the endosomes.

NPY Y₁ receptors mediate ERK1/2-phosphorylation via a calcium-independent pathway.

NPY is also able to stimulate intracellular Ca²⁺ increases via PKC-dependent pathways including the upstream signalling molecules, phospholipase C (PLC) and phospholipase D (PLD). We evaluated the possible involvement of these signalling pathways in NPY-mediated ERK1/2 activation by Y₁ receptors. The results showed that the PKC inhibitor Gö6983 was not able, at the concentrations tested, to block the ERK1/2 phosphorylation induced by 300 pM NPY (Fig. 4B). Likewise, the PLC inhibitor U73122; an inhibitor of PLC activation by G-protein-linked receptors (Thompson et al) or the PLD inhibitor FIPI were both unable to block ERK1/2 activation (Fig. 4A and not shown). Therefore, these results clearly indicate that activation of the human NPY Y₁ receptor induces ERK1/2 activation via a PKC- and PLC-, PLD-independent pathway suggesting that calcium-dependent signalling pathways do not play an important role in this process.

NPY Y₁ receptors mediate ERK1/2-phosphorylation via a PI3Kinase-dependent pathway.

We next assessed for the Y₁ receptor, the contribution of Gβγ subunits to the ERK1/2 activation by using gallein which has been shown to selectively inhibit the interactions between Gβγ and effectors proteins (Lehmann et al. 2008). Pre-treatment with gallein inhibited ERK1/2 activation in a concentration-dependent manner (Fig. 4C).

Earlier studies have shown that the phosphoinositide 3-kinase (PI3K) is a signalling intermediate of the βγ-subunit of G_i-mediated MAP kinase activation (Hawes et al.

1996, Yart et al. 2003). We have pre-treated cells expressing the human NPY Y₁ receptor for 1 h with increasing concentrations of wortmannin, an inhibitor of class I, II and III PI3Ks. Phosphorylation of ERK1/2 by 300 pM NPY was concentration dependently inhibited by the PI3K inhibitor (Fig. 4D) indicating that the signalling pathway conducting to ERK1/2 activation is PI3K-dependent.

Src-family tyrosine kinase activity has also been suggested to be involved in ERK1/2 signalling downstream of activated GPCR receptors **via RTK-transactivation** upstream of PI3K (Luttrell et al. 1996, **Wetzker and Bohmer 2003**, Jeong et al. 2008). To test whether Src kinase is involved in ERK1/2, the Src inhibitor PP2 was used. Cells expressing the human Y₁ NPY receptor were pre-incubated for 1 h with increasing concentrations of PP2 and stimulated with 300 pM NPY. At all concentrations tested, PP2 failed to inhibit ERK1/2 phosphorylation in response to NPY activation (Fig. 4E). Together, the results indicate that upon activation of Y₁ receptors, Src-family tyrosine kinase stimulation is not implicated in ERK1/2 activation.

To assess the role of EGF receptor (EGFR) transactivation in the NPY-induced ERK1/2 phosphorylation, cells expressing the human Y₁ receptor were pre-treated with AG1478, an EGFR inhibitor. After 1 h pre-treatment with various concentration of AG1478, we observed no effect (Fig. 4F) on ERK1/2 phosphorylation in response to 300pM NPY, suggesting that it is unlikely for EGFR to be involved in NPY-mediated activation of ERK1/2.

NPY Y₁ receptors mediate ERK1/2-phosphorylation via an IGFR-dependent but metalloproteinase-independent pathway.

The hypothesis of transactivation of RTKs was further explored. Cells expressing the human Y₁ receptor were pre-treated with AG1024, an specific IGFR inhibitor. After 1 h pre-treatment with various concentration of AG1024, we observed a dose-dependent inhibition (Fig. 5A) on ERK1/2 phosphorylation in response to 300pM NPY. Figure 5B and 5C illustrate the effect of 10μM AG1024 pre-treatment on P-ERK1/2 as compared with vehicle treated cells, with a representative western blot (5B) and the quantification (5C).

The metalloproteinase inhibitor marimastat, that has a large spectrum of action on both matrix-metalloproteinases MMPs but also ADAMs (A Disintegrin And metalloproteases, **Roghani et al. 1999**), was recently shown to inhibit the

transactivation of IGFR receptors in HEK293 cells triggered by the Gs-coupled V2 vasopressin receptor and by the Go/Gq-coupled platelet-activating factor PAF receptor (Oligny-Longpré et al. 2011). In order to test whether NPY induces the shedding of an IGFR ligand through the processing of pre-ligand by plasma membrane metalloproteinases, HEK293 cells expressing the human Y₁ receptor were pre-treated for 1h with 10μM marimastat. We did not observe any inhibition on ERK1/2 phosphorylation in response to 300pM NPY (Fig. 5D). In conclusion, the IGFR is transactivated by Y₁ receptors in HEK293 cells via a Gi/Gbeta-gamma-PI3Kinase-dependent mechanism without the release of a soluble ligand for IGFR.

A similar pathway to ERK1/2 by GPCRs in HEK293 cells

We and other have demonstrated that ERK1/2 is phosphorylated by a number of endogenously expressed GPCRs in HEK293 cells (Michel et al. 2014) and we wanted to address the relative universality of the present pathway. We found that wortmannin, AG1024 but not marimastat were inhibiting the ERK1/2 phosphorylation induced at 5 minutes by the Gq-coupled muscarinic M3 receptor (Figure 6A-D, B-E and C-F respectively) and by the Gs-coupled Adenosine receptor as well (Figure 6G, H, I respectively). This suggests that the MAPkinase pathway governed by Y₁ receptor is also used by endogenously expressed GPCRs in HEK293 cells.

Discussion

In HEK293 cells stably expressing the wild-type NPY Y₁ receptor, activation of this receptor is able to induce a rapid and transient ERK1/2 phosphorylation. The signalling pathway conducting to this activation involves Galphai and Gβ/γ protein subunits. For the first time, we show that MAPkinase activation by Y1 receptors is an internalization-independent pathway and that this receptor can transactivate the IGFR receptor (Figure 7A). We finally show that a similar mechanism of MAPkinase activation through a PI3Kinase-dependent but metalloprotease-independent transactivation of IGFR is used by endogenously expressed Gq- and Gs-coupled receptors in HEK293 cells.

A very interesting mechanism of activation of MAPkinases by GPCRs through transactivation of IGFR was dissected in HEK293 cells recently. It constituted one of the first description of transactivation of IGFR leading to MAPkinase activation by a GPCR (Oligny-Longpré et al. 2011). In this study, the Gs-coupled V2 receptor overexpressed in HEK293 cells was found to mediate an heterotrimeric G-protein-independent transactivation of IGFR independently of PI3kinases (insensitive to wortmannin Charest et al. 2007). It occurs via the release by plasma membrane metalloproteinases of an unidentified soluble agonist of IGFR (inhibition by marimastat). This pathway was dependent on c-Src activation (inhibition by PP2) upstream of IGFR while beta-arrestins were necessary downstream of IGF receptor to trigger MAPkinase activation but not downstream of the activated GPCR themselves (Figure 7B). In a more complex experimental set-up in which EGF receptors were desensitized in HEK293 cells, Gi-coupled-delta-opioid receptors were also found to trigger MAPkinase activation through transactivation of the IGFR via the shedding of an IGF-1-like peptide (Eisinger and Ammer 2010).

The pathway that we are describing now is different than the one described for MAPkinase activation via the vasopressin V2 receptor by several aspects:

MAPkinases activation by NPY Y1 receptor is definitively G-protein dependent, PTX and gallein (Lehmann et al.), respectively specific inhibitors of Galphai and Gbeta-gamma subunits, blocking completely ERK1/2 phosphorylation in a concentration-dependent manner. The pathway that we have unravelled is also clearly insensitive to the c-Src inhibitor PP2 or to the metalloprotease inhibitor marimastat while it is sensitive to wortmannin.

IGFR transactivation by NPY Y1 receptors

On the other hand, we are confirming the previous results that transactivation of IGFR by several GPCRs leads to MAPkinase activation. We demonstrated it using the AG1024 inhibitor, that was one of the few uni-specific kinase inhibitors identified in a recent comprehensive assay of kinase inhibitor selectivity (Anastassiadis et al. 2011). In this same analysis, however, AG1024 was among the 17 uni-specific compounds that inhibited other kinases more potently than the kinases they were intended to target. Indeed, the serine/threonine kinase RIPK2 (Receptor-interacting kinase2) was identified as a much more sensitive target of AG1024. Although RIPK2 becomes phosphorylated upon activation of the Gq-coupled Angiotensin AT1 receptor as described in a recent phosphoproteomic analysis (Christensen et al. 2010), this kinase is an activator of the NF-KB pathway and is a component of signaling in immune responses (ref). Since there is so far no indication that RIPK2 could trigger MAPkinase activation, we rather favour the interpretation that IGFR is the target of AG1024 in our study.

Insulin or IGF ligand binding to the IGFR can activate MAPkinases (Luttrell et al. 1995, Kurihara et al. 2000, Kuemmerle and Murphy 2001). We have observed it in our previous signaling study in HEK293 cells (Michel et al. 2014). The mechanism of ligand-dependent MAPkinase activation of IGFR is not yet clearly determined : beta-arrestins have been proposed to play a role (Oligny-Longpré et al. 2011). Indeed beta-arrestins can serve as platforms to recruit several effectors of the MAPkinase pathway (DeFea for review 2008) and beta-arrestin 1 has been found necessary for MAPkinase activation via the IGFR (Girnita et al. 2007). In this line of thinking, a new concept has emerged in which it is proposed that RTK themselves can transactivate GPCRs, therefore it is not clear whether beta-arrestins are directly interacting with the RTKs or whether interaction with another GPCR is needed in this MAPkinase activation mechanism (Delcourt et al. 2007).

We have not adressed the requirement of beta-arrestins downstream of IGFR because the mechanism of transactivation of IGFR by the Gi-coupled NPY Y1 receptor seems to occur via a ligand-independent activation of IGFR (independent of c-Src and metalloproteases). On the other hand, we have clearly established that recruitment of beta-arrestins and internalization of the NPY Y1 receptor are not necessary for Erk1/2 activation. Two mutants of the NPY Y1 receptor that upon NPY activation are unable to bind beta-arrestins, to activate beta-arrestins and to

internalize into clathrin-coated pits, were unaltered in their amplitudes and kinetics of Erk1/2 phosphorylation (Ouedraogo et al. 2008).

It has been observed in several studies, that in the Gi-coupled receptor and G β γ -mediated MAPkinase signalling pathway, inhibitors of the phosphoinositide 3-kinases (PI3Ks) attenuated the MAP kinase activation demonstrating the requirement of PI3Ks in the intracellular signal transduction pathway through which GPCRs might be able to activate the expression of transcription factors involved in cell growth and proliferation (Hawes et al. 1996).

In our present study a wortmannin-sensitive G β γ -stimulated PI3K activity was required for MAP kinase signalling pathway. Studies indicate that the PI3K- γ isoform of the PI3K family, that is directly activated by G β γ , regulates the signalling from GPCRs (Lopez Ilasaca et al. 1997) especially that of chemokines and chemo attractant receptors (Deane, Procko, Del Prete Delgado-Martin, 2011). But PI3K- β has also been shown to be regulated by G β γ (Yart et al. 2003). The mechanism of transactivation of IGFR by NPY Y1 receptor could thus be similar to the one triggered by the lysophosphatidic acid LPA receptors through transactivation of the EGFR observed in Vero cells (Yart et al. 2003). Indeed, in their study, Yart and colleagues have proposed that PI3K integrates two separate signals from the activated GPCR, both through direct interaction with the released G β γ subunits and at the level of transactivation of the RTK contributing to an amplification of the MAPkinase pathway. In effect, amplification was confirmed using a phosphoproteomics approach, direct EGFR activation of the cells gave rise to weaker phosphorylations of sites phosphorylated by transactivation of the EGFR upon LPA stimulation (Schreiber et al. 2010).

The mechanisms of NPY activation of the MAPkinase pathway described.

While we are the first to propose a transactivation of IGFR and to establish an internalization-independent mechanism, the MAPkinases activation triggered by NPY Y1 receptor that we are describing, is in agreement with previous studies: PTX and wortmannin sensitivity was observed (Keffel et al. 1999, Mullins et al. 2002). Interestingly, a recent investigation has proposed that neuroproliferative effect of NPY in postnatal hippocampal precursor cells is mediated by a MAPkinase pathway occurring downstream of intracellular nitric oxide production, cGMP and the cGMP-dependent protein kinase PKG activation (Alvaro et al. 2008, Cheung et al. 2012).

Further studies are required to investigate the relationship between transactivation of IGFR and nitric oxide production, as was studied for example in the case of angiotensin receptor transactivation of EGFR (Wang et al. 2000) or bradykinin B2 receptor transactivation of vascular endothelial growth factor (Thuringer et al. 2002).

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Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity

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Rapid Transactivation of the Vascular Endothelial Growth Factor Receptor KDR/Flk-1 by the Bradykinin B2 Receptor Contributes to Endothelial Nitric-oxide Synthase Activation in Cardiac Capillary Endothelial Cells*

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GABAB Receptor Activation Protects Neurons from Apoptosis via IGF-1 Receptor Transactivation

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Figures legends

Figure 1

NPY induces ERK1/2 activation in a time- and concentration-dependent manner in human Y₁ receptor (O) expressing HEK293 cells. (A) Time course of ERK1/2 phosphorylation following NPY stimulation. Serum-starved HEK293 cells expressing the NPY Y₁ receptor were incubated with NPY (100 nM) for the indicated times. A representative western blot is displayed together with densitometry analyses of ERK1/2 activation normalized to the maximal response at 5 minutes. In all blots, 20 µg of total protein were analyzed. ERK1/2 phosphorylation was determined using anti-phospho-ERK1/2 antibody. Anti-total-ERK1/2 and anti-actin antibodies were used to control protein concentrations. (B) Concentration-dependence of ERK1/2 phosphorylation. Serum-starved HEK293 cells expressing the NPY Y₁ receptor were incubated for 5 min with increasing concentrations of NPY. (C) Serum-starved HEK293 cells expressing either the NPY Y₁ receptor were pre-treated with varying concentrations of PTX for 18 h followed by 5 min stimulation with NPY (100 nM). (D) Effect of increasing concentrations of MEK inhibitor U0126 on NPY-induced ERK1/2 activation. Serum-starved HEK293 cells expressing the NPY Y₁ receptor were pre-treated with varying concentrations of U0126 for 1 h followed by 5 min stimulation with NPY (300 pM). Values are means ± sem (where not seen, sem is too small to be visible) from 8-9 independent experiments

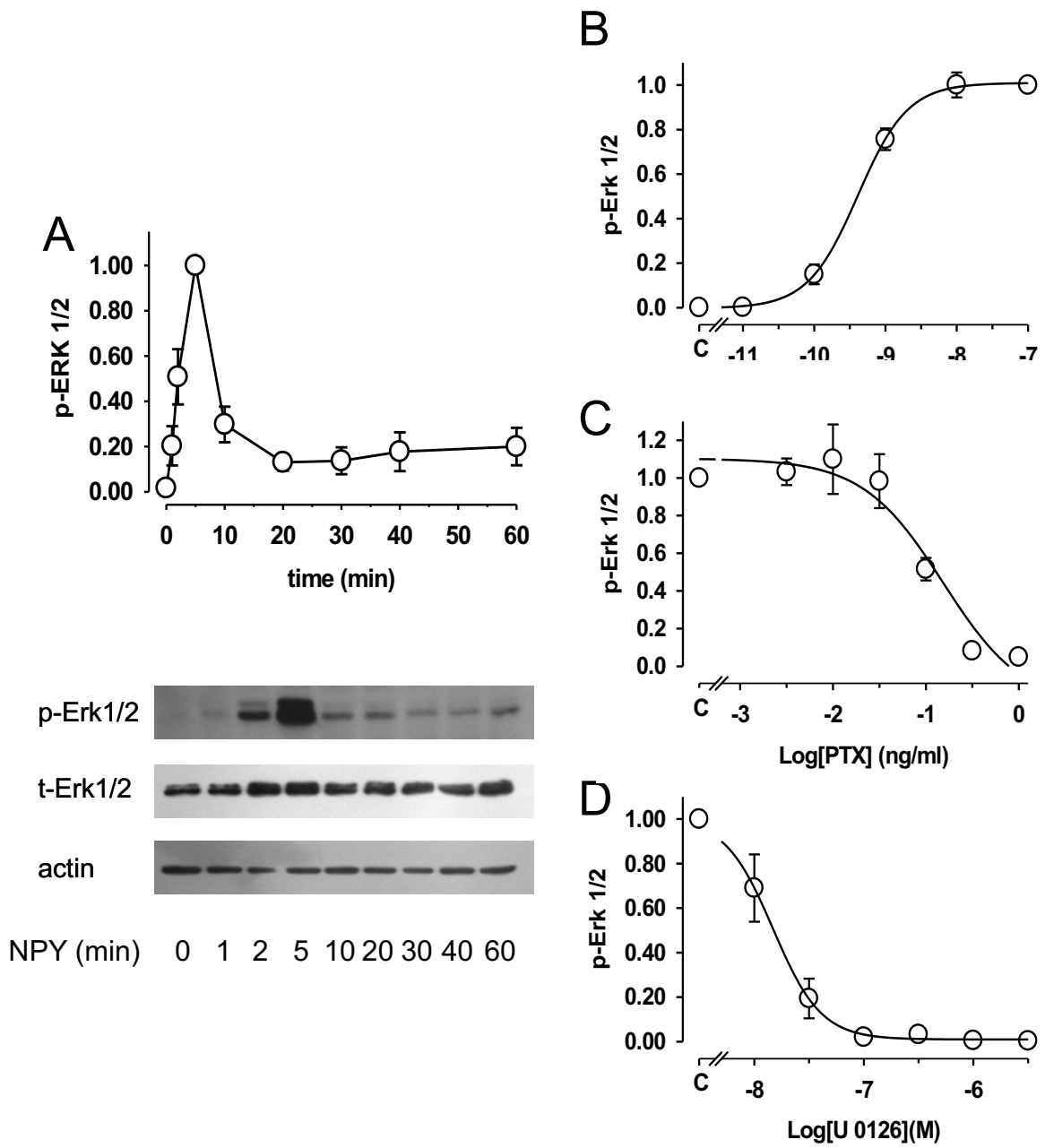


Figure 1

Figure 2

Kinetics of NPY-induced ERK1/2 activation by NPY Y₂ receptor. (A) A representative western blot is displayed illustrating the lower intensities of the ERK1/2 activation by NPY Y₂ receptor as compared to activation by NPY Y₁ receptor. 20 µg of total protein were analyzed. ERK1/2 phosphorylation was determined using anti-phospho-ERK1/2 antibody. Anti-total-ERK1/2 and anti-actin antibodies were used to control protein concentrations. (B) Time course of ERK1/2 phosphorylation following NPY stimulation in human Y₂ (●) receptors expressed in HEK293 cells. Serum-starved HEK293 cells were incubated with NPY (100 nM) for the indicated times, the densitometry analyses of ERK1/2 activation was normalized to the maximal response at 10 minutes. (C) Concentration-dependence of ERK1/2 phosphorylation. Serum-starved HEK293 cells expressing the NPY Y₂ receptor were incubated for 10 min with increasing concentrations of NPY. (D) Serum-starved HEK293 cells expressing either the NPY Y₂ receptor were pre-treated with varying concentrations of PTX for 18 h followed by 10 min stimulation with NPY (100 nM). Shown are densitometry analyses of ERK1/2 activation from 4 to 8 independent experiments.

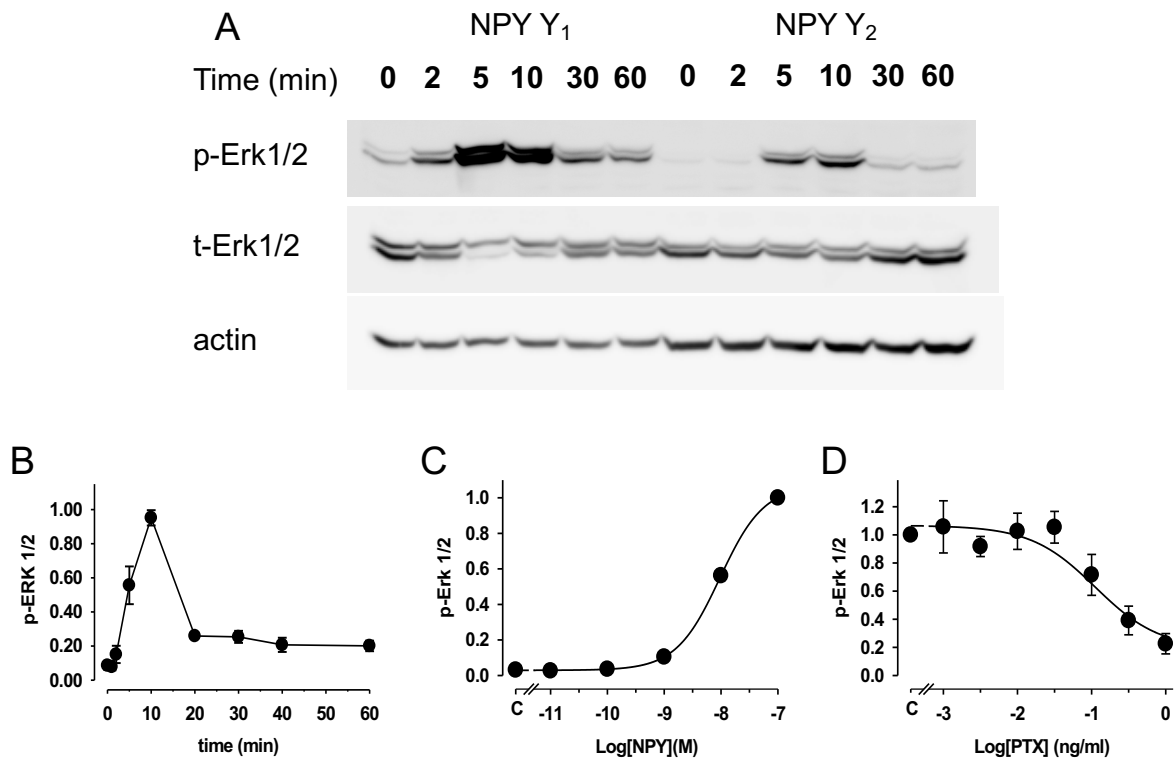


Figure 2

Figure 3

Concentration dependence of ERK1/2 phosphorylation of different mutants of the NPY Y₁ receptor. Time course of ERK1/2 phosphorylation following NPY stimulation in human Y₁ Δ42 (A, □) and Y₁ TDST-A (B, △). Serum-starved HEK293 cells expressing the different NPY receptors were incubated with 100nM NPY at different time points. Values are means ± sem (where not seen, sem is too small to be visible) from 4 to 8 independent experiments.

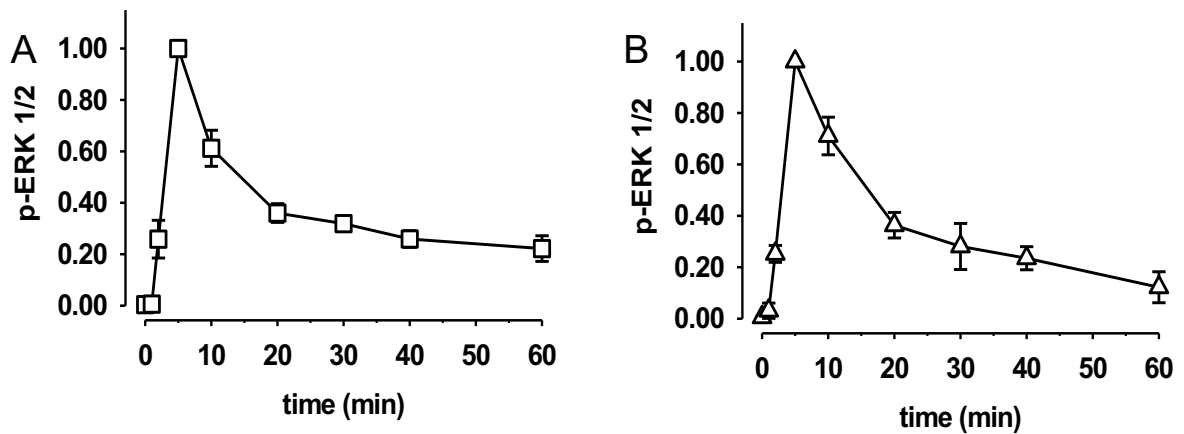


Figure 3

Figure 4

Effect of pre-treatment with various inhibitors on NPY Y₁ receptor induced ERK1/2 activation.

Serum-starved HEK293 cells expressing the NPY Y₁ receptor were pre-treated with varying concentrations of U73112 (PLC inhibitor; A), of Gö6983 (PKC inhibitor, B), of gallein (G β / γ -dependent signalling, C), of the selective PI3K inhibitor wortmannin (D), of PP2 (Src-family tyrosine kinase inhibitor, E) and of AG1478, an EGFR tyrosine kinase inhibitor (F) for 1 h followed by stimulation for 5 min with NPY (300 pM). Values are means \pm sem (where not seen, sem is too small to be visible) from 5-8 independent experiments.

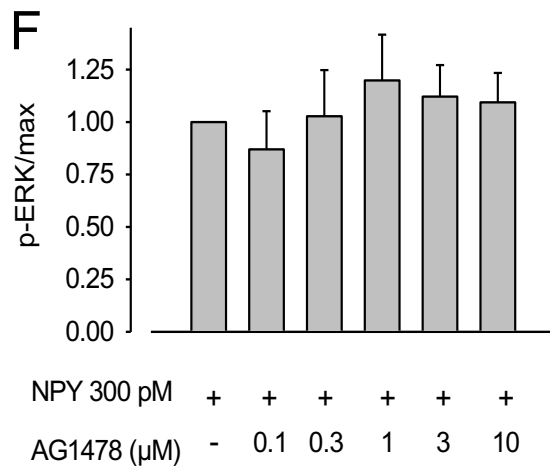
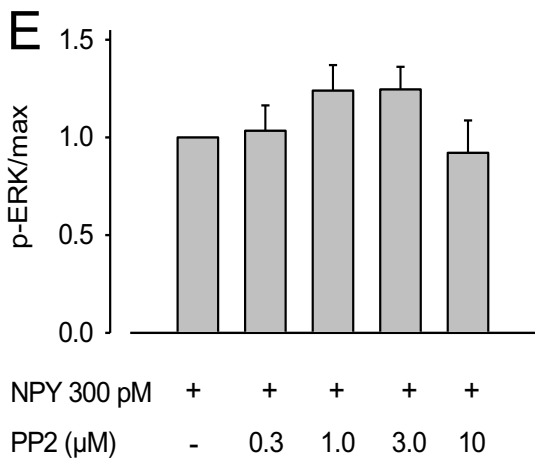
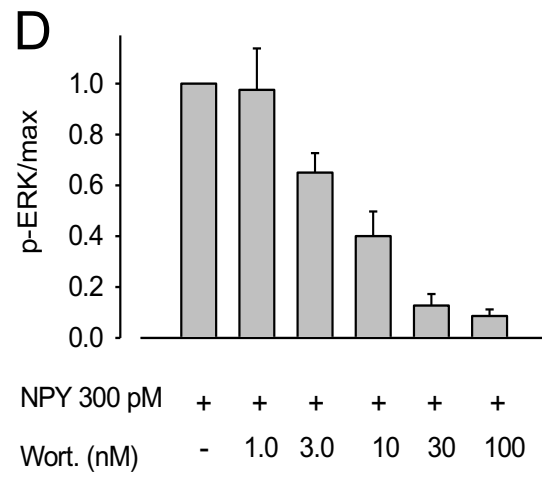
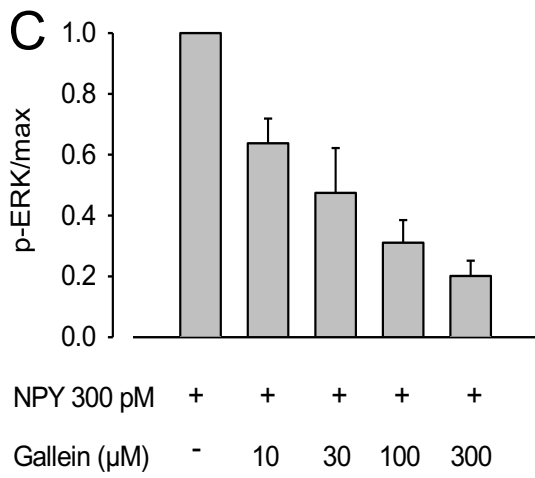
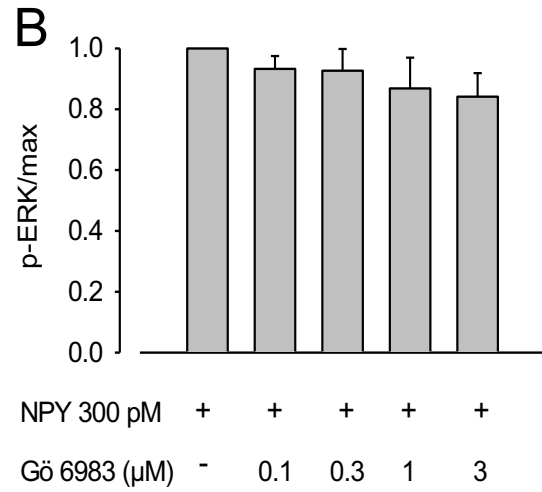
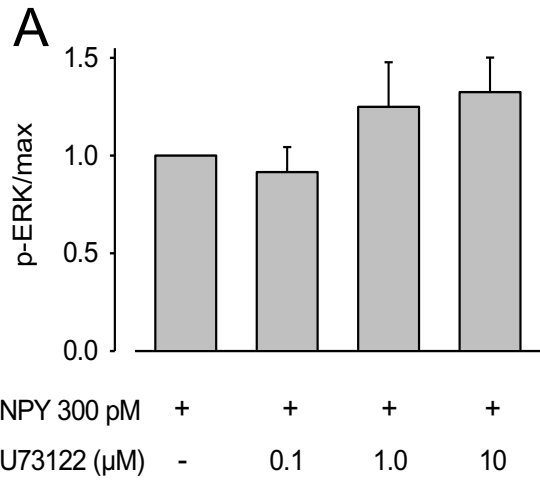


Figure 4

Figure 5

NPY-mediated ERK1/2 activation is dependent on Insulin receptor tyrosine kinase function. Serum-starved HEK293 cells expressing the NPY Y₁ receptor were pre-treated for 1 h with varying concentrations of AG1024 an IGFR tyrosine kinase inhibitor followed by 5 min stimulation with NPY (300 pM). In all blots, 20 μg of total protein were analyzed. Shown are densitometry analyses of ERK1/2 activation. Values are means ± sem from 6 independent experiments.

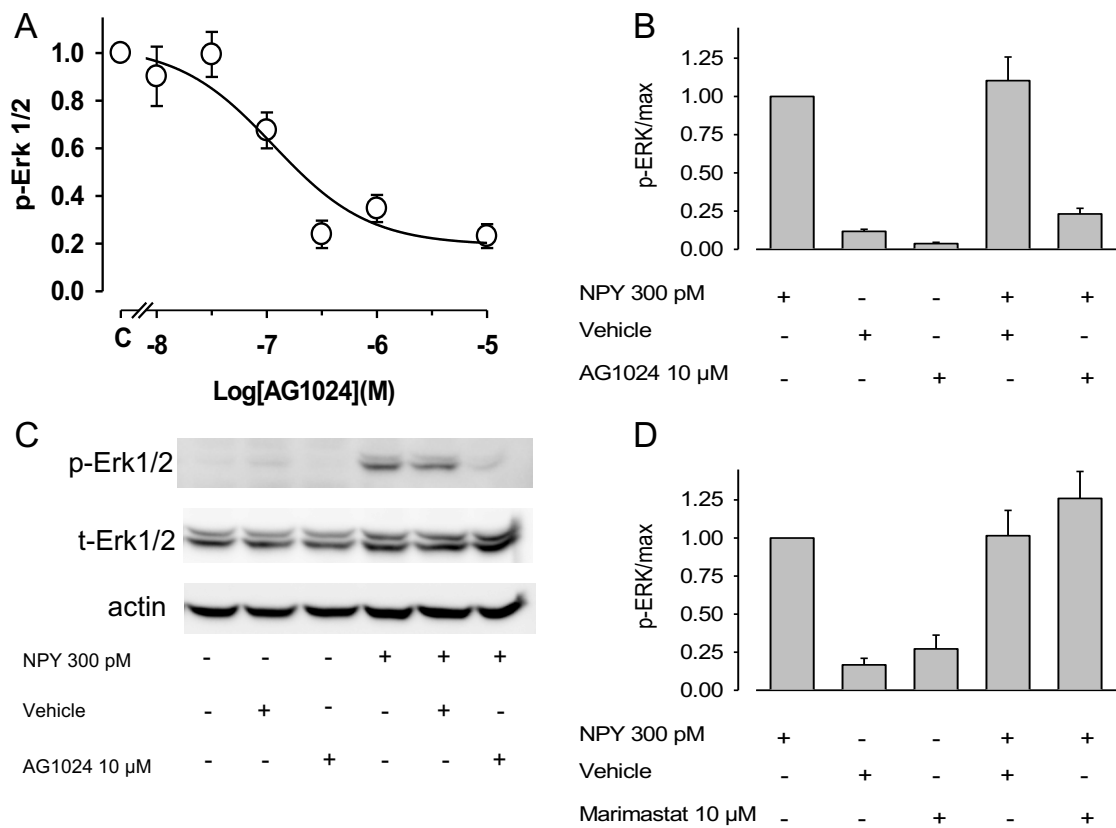


Figure 5

Figure 6

ERK1/2 activation in HEK293 cells by several GPCRs is dependent on a wortmannin-AG1024-sensitive pathway. Representative western blots of serum-starved HEK293 cells were pre-treated for 1 h with Wnt, AG1024 or marimastat followed by 5 min stimulation with Acetylcholine (ACh 1 μ M, endogenous muscarinic M3 receptors, A, B, C respectively). Shown are densitometry analyses of ERK1/2 activation activated by muscarinic M3 receptors (D, E, F) or with agonist of the endogenous Endothelin B receptor (NECA, 30nM G, H, I respectively). Values are means \pm sem from 4 independent experiments.

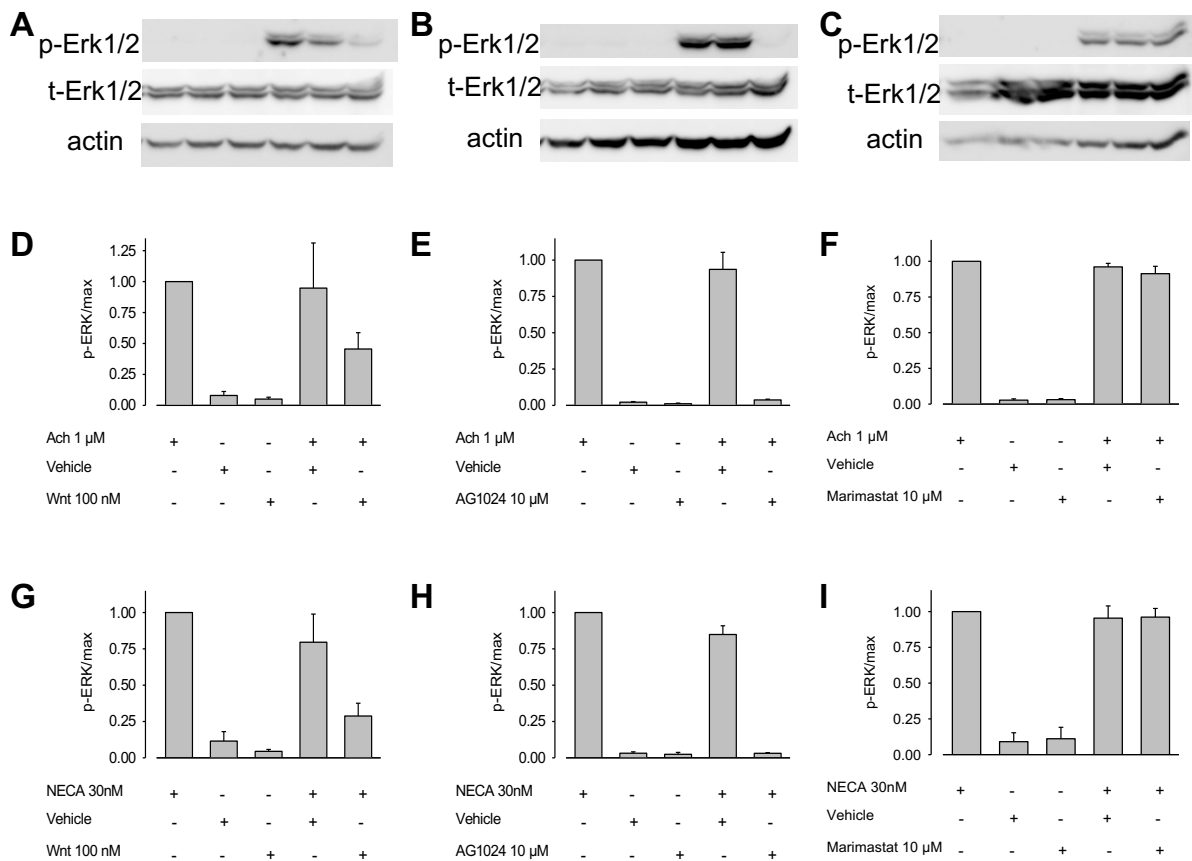


Figure 6