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# Neuropeptide Y receptor mediates activation of ERK1/2 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 neuropeptide Y $Y_1$ receptors, we found that the NPY agonist elicits a transient phosphorylation of the extracellular signal-regulated kinases (ERK1/2). We first show that ERK1/2 activation following $Y_1$ receptor stimulation is dependent on heterotrimeric $G_{i/o}$ since it is completely inhibited by pre-treatment with pertussis toxin. In addition, ERK1/2 activation is internalization-independent since mutant $Y_1$ receptors unable to recruit β-arrestins, can still activate ERK signaling to the same extent as wild-type receptors. We next show that this activation of the MAPK pathway is inhibited by the MEK inhibitor U0126, is not dependent on calcium signaling at the $Y_1$ receptor (no effect upon inhibition of phospholipase C, protein kinase C or protein kinase D) but instead dependent on Gβ/γ and associated signaling pathways that activate PI3-kinase. Although inhibition of the epidermal-growth factor receptor tyrosine kinase did not influence NPY-induced ERK1/2 activation, we show that the inhibition of insulin growth factor receptor IGFR by AG1024 completely blocks activation of ERK1/2 by the $Y_1$ receptor. This Gβ/γ-PI3K-AG1024-sensitive pathway does not involve activation of IGFR through the release of a soluble ligand by metalloproteinases since it is not affected by the metalloproteinase inhibitor marimastat. Finally, we found that a similar pathway, sensitive to wortmannin-AG1024 but insensitive to marimastat, is implicated in activation of ERK signaling in HEK293 cells by endogenously expressed GPCRs coupled to Gq-protein (muscarinic M3 receptors) or coupled to Gs-protein (endothelin ETB receptors). Our analysis is the first to show that β-arrestin recruitment to the NPY $Y_1$ receptor is not necessary for MAPK activation by this receptor but that transactivation of the IGFR receptor is required.



## 1. Introduction

Neuropeptide Y (NPY) is a widely distributed peptide in the central and peripheral nervous system [1]. 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, signaling of GPCRs is mediated by receptor coupling to heterotrimeric G proteins that activate a variety of downstream cellular effectors. The signaling pathway of the NPY family of receptors is known to be coupled to the Gαi-protein, which leads to the inhibition of the adenylyl cyclase and inhibition of cAMP accumulation [2].

The classical paradigm for signaling 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 leads to cell proliferation, neurogenesis or gene transcription, via the phosphorylation of the extracellular signal-regulated protein kinases 1 and 2 [3–5]. GPCRs can mediate ERK1/2 activation by different mechanisms, being able to induce activation of both G protein-dependent and G protein-independent signaling pathways.

Hence, growth-promoting effects of GPCR stimulation have been shown to be mediated through transactivation of growth factor receptor tyrosine kinases (RTKs for the receptors 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) [6]. Different mechanisms of transactivation have been described. These include 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 occur inside the cell, for example, activation of the c-Src-kinase is a generally described mechanism. Alternatively, it has been proposed to occur via direct molecular interactions between RTK and GPCRs or

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via inactivation of protein-tyrosine phosphatases that repress RTK [6–9]. However, transactivation is not an essential requirement in the activation of the signaling cascade induced by GPCR activation [10,11].

During the past decade, evidence has emerged showing that a variety of GPCRs can also couple to other adaptor proteins independently of heterotrimeric G-protein [12,13]. Among these transducers are the scaffolding proteins, β-arrestin-1, and β-arrestin-2, which are cytosolic endocytic adaptors playing an important role in GPCR desensitization, internalization, and trafficking. Upon GPCR activation, they can also function as molecular mediators especially by acting on the ERK1/2 signal transduction pathway [14]. It is increasingly evident that β-arrestins can bind to MAP kinases and facilitate their activation [12].

In previous studies, we have shown that, unlike NPY $Y_2$ receptors, NPY $Y_1$ receptors rapidly internalize through clathrin-coated pits and recycle [15,16]. Upon stimulation by NPY, the endocytic mechanism involves activation of cytosolic β-arrestins and translocation to the plasma membrane. Deletion of the carboxyl-terminal tail of the receptor ($Y_1$ Δ42 receptor) or substitution of key putative phosphorylated residues by alanine in the carboxyl-terminal tail of the receptor ($Y_1$ TDST-A receptor) abolishes both internalization and β-arrestin activation. Likewise, the human NPY $Y_2$ receptor does not internalize in our system and no significant activation of β-arrestin activation is detected [16].

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

## 2. Materials and methods

### 2.1. Cell culture

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, 2 mM glutamine and 1% antibiotics (penicillin/streptomycin) [15]. For activation of the cells, 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 replacing on ice the culture medium by 250 μl of cold RIPA lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with EDTA-free anti-protease and anti-phosphatase cocktails.

cDNA of the human NPY $Y_1$ receptor was kindly provided by Prof. H. Herzog (Garvan Institute, Sydney, Australia). The cloning schemes for the different mutants of the native $Y_1$ receptor used in the present study were previously described [16,17]. All constructions were verified by sequencing and stably transfected cells were established through selective antibiotic selection.

### 2.2. SDS page and western blot analysis

Cell lysates were sonicated three times on ice using a Branson Sonifier at constant power, output = 2.5, and continuous sonication for 1 s. After sonication, insoluble elements were cleared by centrifugation at maximum speed (~14 k) for 5 min at 4 °C. Protein concentration was determined using Bradford reagent. Homogenates of cell lysate were mixed with 4× Laemmli buffer, denatured 5 min at 95 °C, and loaded on a 12% SDS-PAGE gel (20 μg/lane). Protein samples were transferred to immobilon PVDF membranes. Membranes were blocked 1 h (using 5% non-fat dried milk in PBS, pH 7.2, Tween 0.1%), probed overnight with rabbit (polyclonal) anti-ERK1/2 [pTpY185/187] phospho-specific antibody (1:5000, Invitrogen, 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 immune-reactive bands were visualized using Immobilon western chemoluminescent HRP substrate detection reagents (Merck-Millipore) either with hyperfilm or LAS 4000 camera, and bands were quantified using respectively either ImageJ or ImageQuant software (GE-Healthcare). The data were normalized to the maximal phospho-ERK1/2 response for each set of experiments. The statistical significance of the differences between agonist stimulated and agonist stimulated + inhibitor was assessed using one-way ANOVA (Statview). If significant differences between the samples were observed, it was followed by a post hoc Bonferroni test.

### 2.3. Antibodies desorption and second antibodies incubation

Antibodies were stripped from the proteins on the PVDF membrane by 3 incubations of 15 min in a desorbing solution (200 mM HCl, 3% glycine). PVDF membranes were washed thoroughly with PBS, Tween 0.1%, then blocked 1 h using 5% non-fat dried milk in PBS, pH 7.2, Tween 0.1%. PVDF membranes 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 1/10,000. For each western blot, we used actin as a loading control. Mouse monoclonal anti-actin clone C4 1/100,000 came from Merck-Millipore, HRP-goat anti-mouse from GE-Healthcare.

### 2.4. Peptides and chemicals

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

## 3. Results

### 3.1. NPY $Y_1$ receptors mediate ERK1/2-activation via a Gi-dependent pathway

To determine the temporal pattern of ERK1/2 activation mediated by NPY, we examined the kinetics of ERK1/2 phosphorylation following stimulation by NPY in HEK293 cells expressing the $Y_1$ receptor. 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 $Y_1$ receptor (Fig. 1B) with an $EC_{50}$ of 300 pM. In order to assess the role of Gi/o proteins in NPY-mediated ERK1/2 phosphorylation, the effect of selective inhibition of Gi-dependent signaling in cells expressing the human $Y_1$ receptor was examined. PTX pre-treatment inhibited ERK1/2 phosphorylation induced by 100 nM NPY in a concentration manner with an $IC_{50}$ of 150 pg/ml, indicating use of Gi/o proteins in the ERK signaling pathway (Fig. 1C). In all the following experiments we used the EC50 value of NPY concentration to test which pathway mediates MAPK activation. The inhibitor of the mitogen-activated protein (MAP) kinase/ERK kinase (MEK), U0126, abolished ERK1/2 phosphorylation in response to 300 pM NPY in a concentration-dependent manner, with an $IC_{50}$ of 15 nM, indicating a conventional mechanism through MEK directly upstream of ERK1/2 activation (Fig. 1D).