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β-arrestin1 phosphorylation by GRK5 regulates G protein-independent 5-HT_4 receptor signalling

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G protein-coupled receptors (GPCRs) have been found to trigger G protein-independent signalling. However, the regulation of G protein-independent pathways, especially their desensitization, is poorly characterized. Here, we show that the G protein-independent 5-HT_4 receptor (5-HT_4R)-operated Src/ERK (extracellular signal-regulated kinase) pathway, but not the G_s pathway, is inhibited by GPCR kinase 5 (GRK5), physically associated with the proximal region of receptor' C-terminus in both human embryonic kidney (HEK)-293 cells and colliculi neurons. This inhibition required two sequences of events: the association of β–arrestin1 to a phosphorylated serine/threonine cluster located within the receptor C-t domain and the phosphorylation, by GRK5, of β–arrestin1 (at Ser^{412}) bound to the receptor. Phosphorylated β-arrestin1 in turn prevented activation of Src constitutively bound to 5-HT_4Rs, a necessary step in receptor-stimulated ERK signalling. This is the first demonstration that β-arrestin1 phosphorylation by GRK5 regulates G protein-independent signalling.

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Introduction

G protein-coupled receptor (GPCRs) have been first characterized for their ability to activate G proteins and to engage G protein-dependent signalling pathways. The desensitization process, which controls the duration and the intensity of these G protein-mediated signals, has been extensively characterized during the last two decades. It requires GPCR kinase (GRK)-dependent phosphorylation of GPCRs, their uncoupling from the G protein, and subsequent recruitment of β-arrestins (β-arrs). This latter event promotes receptor endocytosis, precluding further G protein activation. Recently, additional signalling pathways have been discovered in addition to the classical G protein-dependent signalling responsible for second messenger generation. For instance, activation of the extracellular signal-regulated kinase (ERK) (Shenoy *et al*, 2006), Src (Heuss *et al*, 1999; Barthet *et al*, 2007; Sun *et al*, 2007) and phospholipase D pathways (Cao *et al*, 1996) by ligands of certain GPCRs occurs independently of G protein activation and the generation of second messengers (see, for reviews, Heuss and Gerber, 2000; Bockaert *et al*, 2004a; Kim *et al*, 2005; Lefkowitz and Shenoy, 2005; Premont and Gainetdinov, 2007). The regulation and desensitization of G protein-independent signalling pathways are poorly characterized. In particular, a fundamental issue is to determine whether the desensitization of G protein-dependent and -independent signals are governed by common or distinct molecular mechanisms, and whether key molecules involved in the desensitization of G protein-dependent signalling such as GRKs and β-arrs are also important for G protein-independent signalling desensitization.

A few reports have described a role of GRKs in the regulation of G protein-independent ERK signalling. GRK2, the major GRK involved in recruitment of β-arrs by GPCRs, is also able to inhibit G protein-independent, β-arr-dependent ERK activation by angiotensin II type $_{1A}$ (AT_{1A}), V_2 vasopressin (V_2) and follicle-stimulating hormone (FSH) receptors (Hunton *et al*, 2005; Kim *et al*, 2005; Ren *et al*, 2005; Kara *et al*, 2006). In contrast, GRK5/6 promote rather than inhibit G protein-independent, β-arr-mediated ERK activation by AT_{1A}, V_2, β_2-adrenergic (β_2-AR) and FSH receptors (Kim *et al*, 2005; Ren *et al*, 2005; Kara *et al*, 2006; Shenoy *et al*, 2006). In addition to their role in desensitization of G protein-dependent signals, both β-arr1 and β-arr2 can serve as platforms for the recruitment of specific signalling proteins. For instance, they have been identified as essential components involved in the activation of the ERK signalling pathway mediated by several GPCRs (DeWire *et al*, 2007). However, for certain GPCRs such as AT_{1A} receptor, β-arr1 can act as a dominant-negative inhibitor of β-arr2-dependent, receptor-operated ERK activation (Ahn *et al*, 2004a, 2004b).

The 5–HT_4R is a GPCR, which signals through both G protein-dependent and -independent pathways. The major G protein-dependent pathway engaged by 5–HT_4R is a G_s/cAMP/PKA pathway, which is desensitized by GRK2

(Barthet *et al*, 2005). We have recently described a G protein-independent pathway activated by this receptor (the Src/ERK pathway) whose desensitization mechanism is unknown (Barthet *et al*, 2007).

Here, we investigated the regulation of the G protein-independent signalling of 5–HT$_4$R by GRKs. We showed that the G protein-independent Src/ERK activation by 5–HT$_4$R was inhibited by GRK5. This negative regulation was specific of the G protein-independent Src/ERK pathway, as GRK5 only played a marginal role in 5–HT$_4$Rs/G$_s$ uncoupling (Barthet *et al*, 2005). Moreover, we demonstrated that GRK5 inhibited 5–HT$_4$R-mediated Src/ERK activation through direct phosphorylation of β-arr1 bound to a S/T cluster located within its C-terminal domain (C-t).

Results

Inhibition of the 5-HT$_4$R-stimulated G protein-independent Src/ERK pathway by GRK5

We earlier identified GRK2 as the major GRK responsible for the desensitization of the 5–HT$_4$R-mediated G$_s$ pathway (Barthet *et al*, 2005). Here, we have searched for a GRK able to inhibit the 5-HT$_4$R-mediated G protein-independent Src/ERK pathway without affecting the 5-HT$_4$R-mediated Gs pathway.

We focused on GRK5 because its membrane recruitment at the plasma membrane does not depend on G proteins. Co-expression of GRK5 with 5–HT$_4$R in human embryonic kidney (HEK)-293 cells did not significantly reduce cAMP formation evoked by 5-HT (Supplementary Figure S1A). In contrast, GRK5 expression strongly attenuated phosphorylation of ERK induced by 5- and 30-min exposures to 10 μM 5-HT (Figure 1; Supplementary Figure S1B). Moreover, GRK5 expression prevented 5-HT-induced activation of Src, as assessed by its phosphorylation on Tyr^{416} (p-Y^{416}-Src, Figure 1). These inhibitory effects depended on GRK5 activity as expression of a kinase-dead GRK5 mutant (K^{215}R) did not reduce receptor-mediated phosphorylation of Src and ERK (Figure 1). In contrast, expression of GRK5 (K^{215}R) enhanced ERK phosphorylation ($+30\pm11\%$, $n=6$, Figure 1B). This observation likely reflects a dominant negative effect of overexpressed GRK5 (K^{215}R) on the inhibition of the ERK pathway elicited by endogenous GRK5.

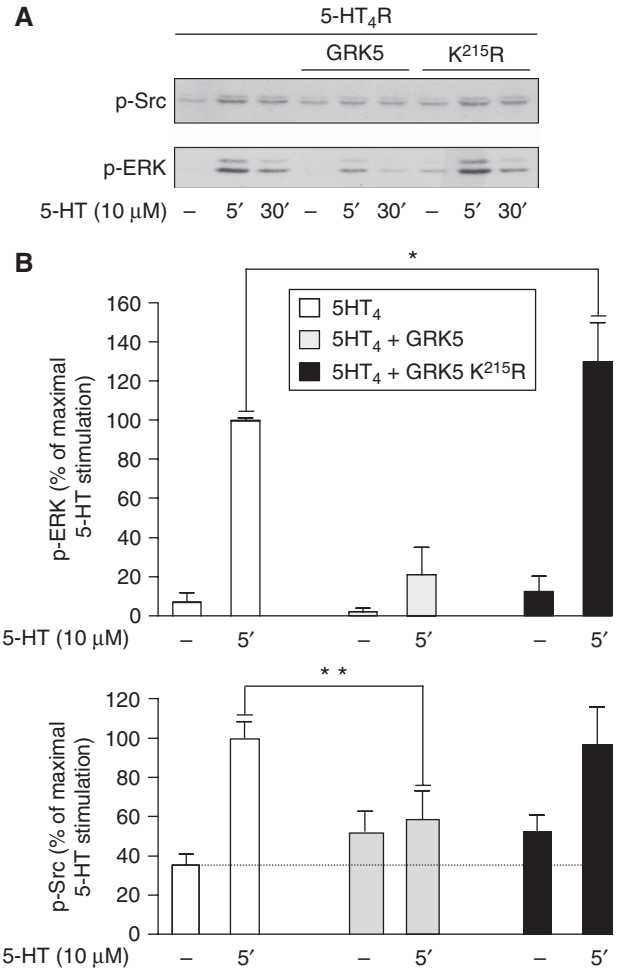

Figure 1 GRK5 suppresses the 5-HT$_4$R-mediated activation of the Src/ERK pathway. (**A**) HEK-293 cells transiently expressing 5-HT$_4$Rs were co-transfected or not with GRK5 or the kinase-dead mutant GRK5 (K^{215}R) and serum starved before 5-HT (10 μM) exposure for the indicated time. HEK-293 cells were lysed in SDS sample buffer, subjected to SDS–PAGE. ERK and Src activation were analysed by western blotting using antibodies against phospho-Thr^{202}/Tyr^{204}-ERK1/2 (p-ERK) and phospho-Tyr^{416}-Src (p-Y^{416}-Src). Total ERK1/2 and total Src were revealed on the same blot with polyclonal antibody recognizing ERK and Src independently of their phosphorylation sites (not shown). Note that total ERK and Src were not affected in all experiments. The data are representative of a series of blots performed in the same conditions. (**B**) P-ERK and p-Y^{416}-Src expressed as percentage of maximal 5-HT stimulation ± s.e.m., represented by densitometric quantification of western blot performed from four different experiments. $^*P<0.05$ or $^{**}P<0.01$ versus corresponding values measured in cells transfected with WT 5-HT$_4$R alone.

Role of a S/T cluster within the 5-HT$_4$R C-terminus on GRK5-mediated inhibition of the receptor-operated ERK pathway

The 5-HT$_4$R C-t encompasses several S/T residues, which are potential GRK5 phosphorylation sites. We first examined the possible role of these residues in GRK5-mediated inhibition of ERK by generating several 5-HT$_4$R truncated mutants (Figure 2A). GRK5 failed to inhibit ERK phosphorylation induced by 5-HT in cells expressing either the Δ329 mutant lacking the entire C-terminus or the Δ346 mutant lacking a S/T cluster (residues 347–355, Figure 2A). Deletion of the S/T cluster likewise prevented inhibition, by GRK5, of Src phosphorylation elicited by 5-HT (Figure 2B). GRK5 still inhibited phosphorylation of ERK induced by 5-HT in cells expressing either the truncated receptor lacking its PDZ ligand (ΔSCF, Figure 2A) with a putative phosphorylated site (S^{385}) or the Δ358 mutant comprising the S/T cluster and lacking the last four scattered S/T residues, but not in cells expressing the corresponding truncated receptor in which all S/T residues of the cluster have been mutated into alanine (Δ358-Ala, Figure 2A).

Collectively, these results indicate that the S/T cluster (residues 347–355), which was previously shown to bind to β-arr (Barthet *et al*, 2005), constitutes a key molecular determinant implicated in the regulation of ERK activation by GRK5. Further supporting its essential role in the regulation by GRK5 of the ERK pathway, progressive deletion of the S/T residues within the cluster concomitantly reduced the GRK5-mediated inhibition of ERK phosphorylation (Supplementary Figure S2).

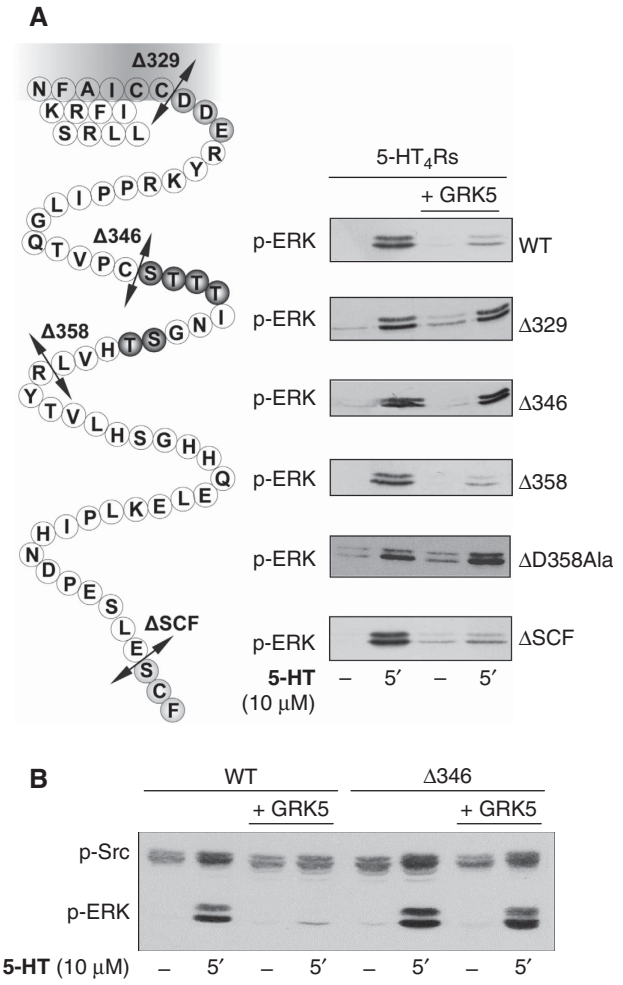

Figure 2 A Serine/Threonine cluster within the 5-HT$_4$R C-t is essential for inhibition, by GRK5, of receptor operated ERK signalling. (**A**) Topology of 5-HT$_4$R C-t domain. The successive points of truncation are illustrated represented on the left with an arrow. HEK-293 cells were transiently transfected with WT 5-HT$_4$R or the corresponding truncated or mutated receptors (Δ329, Δ346, Δ358, Δ358Ala) in combination with or without GRK5. Identical expression levels of the transfected constructs were controlled by ELISA. Cells expressing the indicated receptors were treated or not with 10 μM 5-HT for 5 min. ERK activation was analysed by immunoblotting with p-ERK1/2 antibody. (**B**) HEK-293 cells were either transfected with a plasmid encoding Myc-tagged WT 5-HT$_4$R or Myc-tagged Δ346 alone or co-transfected with GRK5. They were challenged with 10 μM 5-HT for 5 min. Total lysates were analysed by sequential immunoblotting, using p-Y^{416}-Src, p-ERK1/2 antibodies.

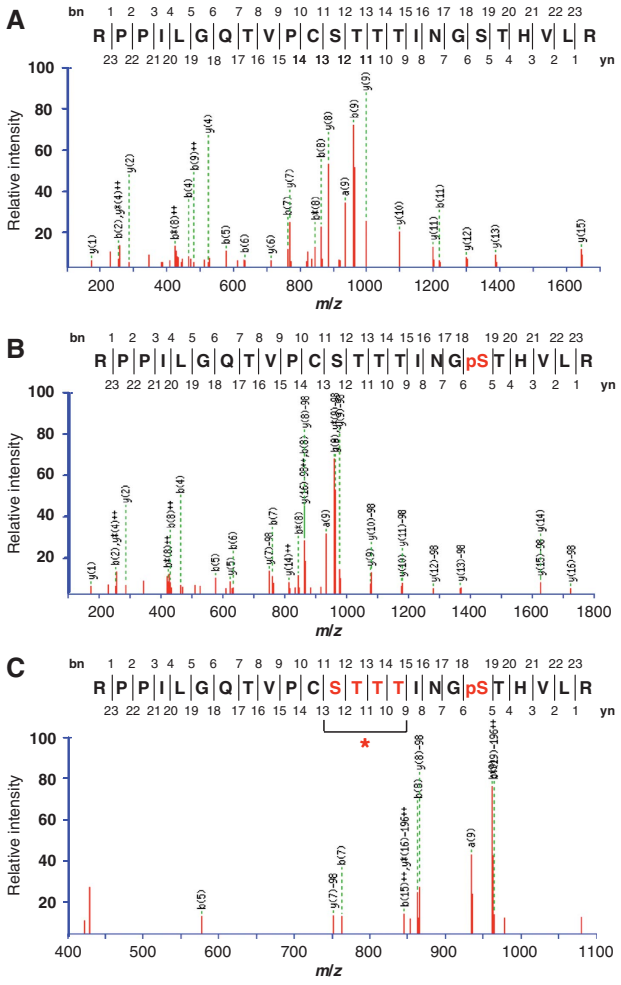

Figure 3 Analysis of 5-HT-dependent phosphorylation of 5-HT$_4$R by tandem mass spectrometry. HEK-293 cells co-transfected with HA-tagged 5-HT$_4$R and GRK5 constructs were treated with 5-HT (10 μM, 10 min). Immunoprecipitated receptors were digested with trypsin and peptides were analysed by nano-LC FT MS/MS. MS/MS spectra resulting from higher energy collisional dissociation (HCD) fragmentation of the non-phosphosphorylated, monophosphorylated and bi-phosphorylated versions of the R^{336}-R^{359} peptide are depicted in (**A**), (**B**) and (**C**) respectively. The three peptides have respective mascot scores of 66, 65 and 59. (C) The bracket indicates that one residue of the S^{347}TTT^{350} motif is phosphorylated. Its exact position could not be determined by MS/MS.

We then analysed agonist-dependent phosphorylation sites on the receptor by tandem mass spectrometry (MS/MS). HEK-293 cells expressing HA-tagged 5-HT$_4$R and GRK5 constructs were treated or not with 5-HT for 10 min. Receptors were then immunoprecipitated on anti-HA agarose beads. Immunoprecipitated receptors were resolved by SDS–PAGE and digested in-gel with trypsin. In control cells (not treated with 5-HT), analysis of duplicate samples revealed the presence of a non-phosphorylated form of the peptide comprising the S/T cluster (R^{336}-R^{359}). In contrast, several versions of the peptide were identified in cells exposed to 5-HT: the non-phosphorylated one and three phosphorylated forms with one, two and three phosphates attached, respectively. Loss of phosphate on fragmentation indicated phosphorylation of S^{354} in the mono-phosphorylated peptide (Figure 3B) and the presence of an additional phosphorylated residue within the S^{347}TTT^{350} motif in the peptide with two phosphates attached (Figure 3C). The tri-phosphorylated peptide incorporated an additional phosphate within the S^{347}TTT^{350} motif (not illustrated). These results indicated sequential phosphorylation of the peptide, first on S^{354} and then in the S^{347}TTT^{350} motif.

Physical association of GRK5 with 5-HT$_4$Rs, a necessary step in GRK5-mediated inhibition of 5-HT$_4$R-operated ERK signal

We next determined whether GRK5 physically interacted with 5-HT$_4$R. GRK5/5-HT$_4$R interaction was first investigated in an *in vitro* binding assay using purified GRK5 and recombinant

S-tagged receptors immobilized on a S-protein agarose column (Baneres *et al*, 2005). 5-HT induced association of GRK5 with 5-HT$_4$R, as assessed by the lack of GRK5 detection in the flow-through fraction when 5-HT was present (FT, Figure 4A). Moreover, a greater amount of GRK5 was co-eluted with the receptor compared with sample not treated with 5-HT (E, Figure 4A). In contrast, leukotriene B4 failed to induce association of GRK5 with immobilized, recombinant BLT1 receptors (Figure 4A), indicating that GRK5 does not interact with every GPCR on activation by its cognate agonist.

These results indicate that 5-HT promotes *direct* interaction of GRK5 with 5-HT$_4$Rs. We then explored the molecular determinants involved in the interaction of GRK5 with 5-HT$_4$Rs in HEK-293 cells by co-immunoprecipitation. GRK5 co-immunoprecipitated with 5-HT$_4$R and the amount of GRK5 was enhanced on 5-HT exposure (Figure 4B). GRK5 did not co-immunoprecipitate with the Δ329 truncation mutant lacking the entire C-terminus in the presence of 5-HT (Supplementary Figure S3), whereas the Δ346 mutant that lacks the S/T (347–355) cluster phosphorylated by GRK5 (Supplementary Figure S2) still interacted with GRK5 (Figure 4B). Thus, GRK5 can bind to 5-HT$_4$Rs without the presence of the S/T cluster substrates. However, note that 5-HT did not stimulate GRK5 binding to the Δ346 mutant.

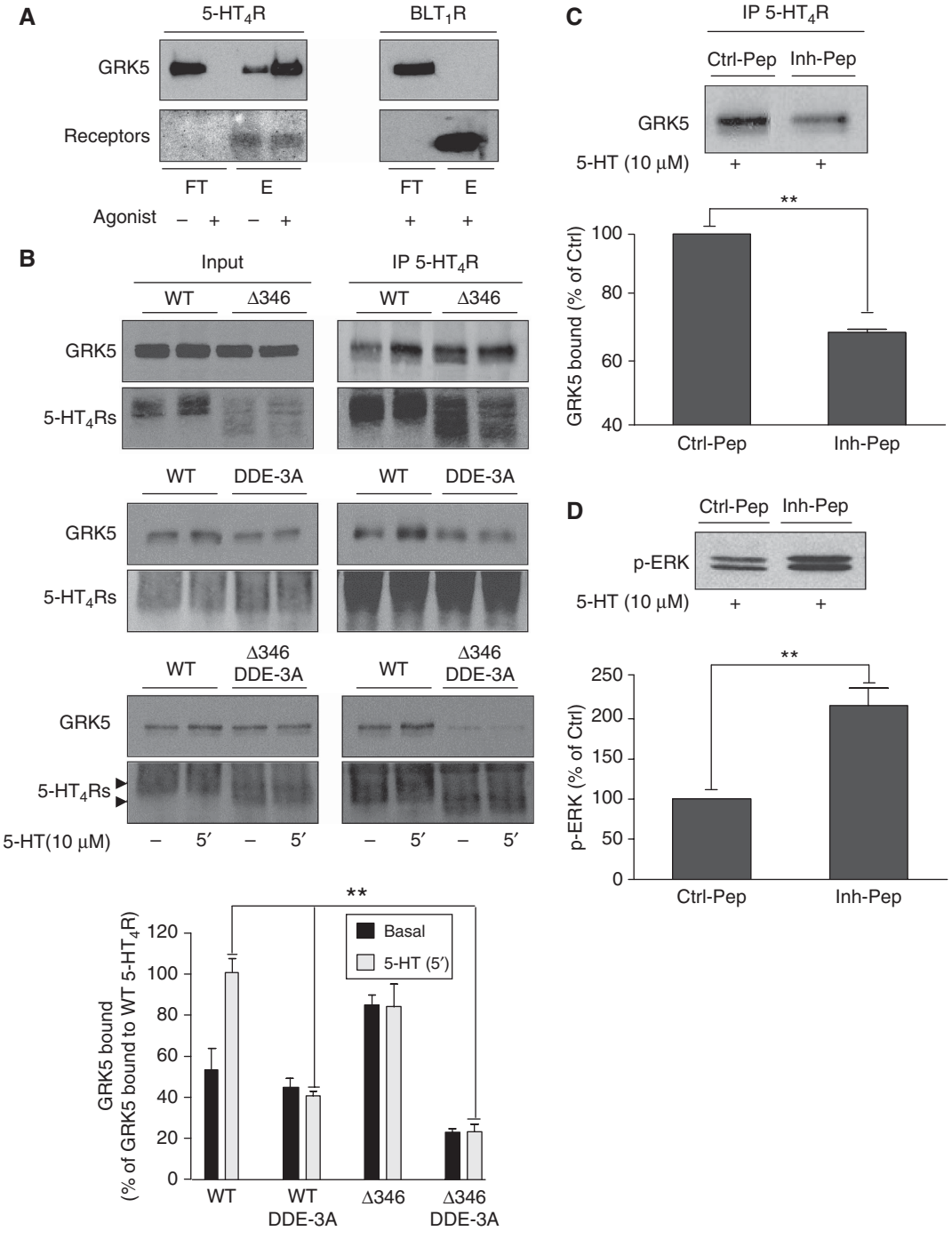

Figure 4 See over for legend.

An earlier study has shown that acidic residues located upstream GRK phosphorylation sites participate in the recruitment of GRKs by several GPCRs (Berrada *et al*, 2000). A series of acidic residues located in the juxtamembrane region of 5-HT$_4$R C-t ($D^{330}DE^{332}$) was likewise critical for GRK5 recruitment by 5-HT$_4$Rs. Indeed, mutation of these residues into alanine in WT and Δ346 receptors strongly reduced GRK5 recruitment (Figure 4B). Collectively, these results identified the proximal region of the 5-HT$_4$R C-t (amino-acids 330–346) as the GRK5 binding sequence and the $D^{330}DE^{332}$ motif as the most important determinant. To further explore the role of GRK5/5-HT$_4$R interaction, we synthesized an interfering peptide (Inh-Pep for inhibitor peptide) comprising the GRK5 binding site (residues 330–345, Figure 2A). The peptide was N-terminally fused to the transduction domain of the TAT protein from HIV Type 1 to allow its intracellular delivery (Aarts *et al*, 2002). Treatment of HEK-293 cells co-transfected with 5-HT$_4$R and GRK5 with Inh-Pep (10 μM, 1 h) reduced 5-HT$_4$R/GRK5 co-immunoprecipitation (Figure 4C). In non-GRK5 transfected cells, Inh-Pep increased by 50% the 5-HT$_4$R-stimulated ERK phosphorylation compared with the 5-HT$_4$R-stimulated ERK phosphorylation measured in cells treated with a control TAT-derived peptide (including the 5-HT$_{2C}$ receptor C-t) (Figure 4D). This effect likely reflected competition of Inh-Pep with endogenously GRK5 and reversal of its inhibitory effect.

β-arrestin1 is essential for GRK5-mediated inhibition of the 5-HT$_4$R-operated Src/ERK pathway

Our data indicated that association of GRK5 with the proximal region of 5-HT$_4$R C-t (sequence 330–346) was required but was not sufficient. Phosphorylation of S/T residues within the S/T cluster (residues 347–355) was also essential. As this cluster is known to bind to β-arrs (Barthet *et al*, 2005), we explored a possible role of β-arrs in the GRK5 effect. WT receptor interacted with both endogenous β-arr1 and β-arr2 (Figure 5A). This association was increased by 5-HT (Figure 5A). The Δ346 mutant slightly interacted with endogenous β-arr2, but not with β-arr1. Furthermore, the association of β-arr2 with the mutant was not increased on 5-HT exposure (Figure 5A; Supplementary Figure S4). Transfection of HEK-293 cells with siRNA directed against β-arr1, which strongly decreased β-arr1 expression compared with control siRNA-transfected cells, markedly impaired GRK5-mediated inhibition of the 5–HT$_4$R-operated activation of the ERK pathway (Figure 5B and C). To further confirm that β-arr1 was essential for GRK5-mediated inhibition of ERK signalling, we transfected mouse embryonic fibroblasts (MEFs) lacking β-arr1 and β-arr2 (β-arr1/2$^{-/-}$) with 5–HT$_4$R. As these cells endogenously express various 5-HTRs, 5-HT$_4$Rs were stimulated by BIMU8 (a selective 5–HT$_4$R agonist) instead of 5-HT. Overexpression of GRK5 did not inhibit the 5-HT$_4$R-mediated activation of the ERK pathway in β-arr1/2$^{-/-}$ MEF cells (Figure 6). This inhibition was rescued by co-transfecting cells with β-arr1 and to a much lesser extent with β-arr2.

The phosphorylated β-arrestin1 (p-S^{412}-β-arr1) is required for the GRK5-mediated inhibition of 5-HT$_4$R-operated ERK signalling

We have shown that GRK5-mediated inhibition of 5-HT$_4$R-operated ERK signalling depends on phosphorylation of the S/T cluster (residues 347–355) of 5-HT$_4$R C-t and on β-arr1 binding to the receptor. However, expression of GRK5 did not further increase the association of β-arr1 to the receptor on 5-HT exposure (Figure 7A and B). Thus, the mechanism by which GRK5 inhibited the 5-HT$_4$R-induced ERK signalling (which is dependent on β-arr1) was obviously not a simple 'increase' in β-arr1 recruitment by the S/T cluster (residues 347–355).

Insulin-induced desensitization of ERK activation by Gα_i-coupled receptors has been shown to require phosphorylation of β-arr1 at S^{412} (p-S^{412}-β-arr1). ERK itself was implicated in insulin-induced β-arr1 phosphorylation (Hupfeld *et al*, 2005), consistent with earlier findings, indicating that

Figure 4 A direct interaction between GRK5 and 5-HT$_4$R is necessary for ERK pathway regulation. (**A**) Recombinant purified His-tag 5-HT$_4$R obtained as described earlier (Baneres *et al*, 2005) were incubated with purified full-length GRK5 (Cell Signalling Technology) in the presence or absence of 5-HT (10 μM). The western blot analysis with the antibody against GRK5 or anti-His-tag (receptor) showed the fractions eluted from the S-agarose column. Fraction FT: in flow-through fractions the unbound proteins were detected. Fraction E: the receptor immobilized on the column and its tightly associated proteins were recovered in this fraction E (see Materials and methods). His-Tag-BLT1, which did not interact with GRK5, is used as control (right part). − and + indicate the absence or the presence of the agonists, 5-HT for 5-HT$_4$R at 10 μM and LTB$_4$ at 10 μM for BLT1R. The control has been carried out only in the presence of the agonist LTB$_4$. (**B**) HEK-293 cells were either transfected with a plasmid encoding Myc-tagged WT 5-HT$_4$R or different Myc-tagged mutants, co-transfected with GRK5. $DDE^{330-332}$ indicated by grey circles in Figure 2A, represent the putative binding site of GRK5. Cells co-expressing the indicated receptors and GRK5 were challenged with 10 μM 5-HT for 5 min. Receptors were immunoprecipitated using polyclonal-anti-Myc antibody. Co-precipitated GRK5 was analysed by western blotting by using the antibody against GRK5 a gift from Dr RJ Lefkowitz (Duke University Medical Center, Durham, NC). Immunoprecipitated proteins were analysed by western blotting using the monoclonal anti-Myc antibody. On the left part, inputs represent 5% of the total protein amount used in immunoprecipitation. Quantification of GRK5 bound to the receptor was performed by densitometry using the NIH Image Sofware. Data are means ± s.e.m. of results obtained in four independent experiments. $^{**}P<0.01$ versus corresponding values measured in experiments performed from cells expressing WT 5-HT$_4$Rs. (**C**) The 5-HT$_4$R C-t peptide (Inh-Pep) reduced the association of GRK5 with the receptor. The sequence (330 to 346) located on 5-HT$_4$R-C-t (see Figure 2A) (DDERYKRPPILGQTVPC) fused to the transduction domain of TAT protein (YGRKKRRQRRR) was used as inhibitor peptide. HEK-293 cells co-transfected with Myc-tagged-WT 5-HT$_4$R and GRK5 were treated with either 10 μM of TAT-Inh-Pep or TAT-Ctrl (Ctrl-Pep) (the C-t residues of 5-HT$_{2c}$-C-t VNPSSVVSERISSV fused with TAT protein (YGRKKRRQRRR) for 1 h with 10 μM 5-HT for 5 min. Receptors were immunoprecipitated using the polyclonal anti-Myc antibody. Immunoprecipitated receptor was detected with the monoclonal anti-Myc antibody. Co-precipitated GRK5 was analysed by western blotting using the anti-GRK5 antibody. Intensities of the bands in immunoblots were measured by densitometry analysis using Image J software. Data are means ± s.e.m. of results obtained in four independent experiments. $^{**}P<0.01$ versus corresponding values obtained from cells treated with the control peptide (Ctrl-Pep). (**D**) The 5-HT$_4$R C-t peptide (Inh-Pep) increased the 5-HT$_4$R induced ERK signalling. HEK-293 cells were pre-incubated for 1 h with either 10 μM TAT-peptides (Inh-Pep) or a control peptide (see C) before the 5-min challenge (10 μM 5-HT). Cells were then lysed in SDS sample buffer and subjected to SDS–PAGE. ERK activation was analysed by western blot using the polyclonal antibody against phospho-Thr^{202}/Tyr^{204}-ERK1/2. Illustrated data are representative of four blots performed in different sets of cultured cells. The histogram is the means ± of densitometric quantification of four western blots using Image J software.

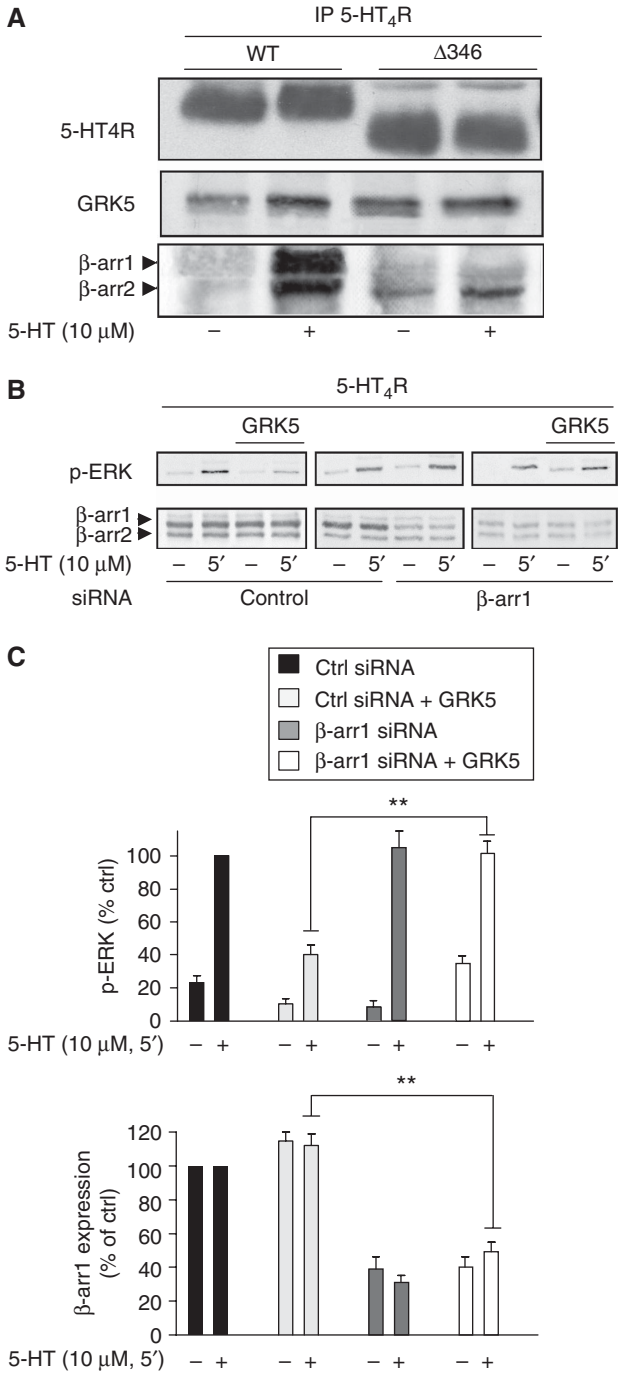

Figure 5 Role of β-arrestins in the inhibition, by GRK5, of the 5-HT$_4$R-activated Src/ERK pathway (**A**) Activated WT 5-HT$_4$R and Δ346 mutant differentially interact with endogenous β-arr1. HEK-293 cells co-transfected with either Myc-tagged Δ346 or Myc-tagged WT 5-HT$_4$R and GRK5 were challenged with 10 μM 5-HT for 5 min. Receptors were immunoprecipitated using the polyclonal anti-Myc antibody. Co-precipitated β-arr 1/2 were analysed by western blotting by using the anti-β-arr A1CT antibody, which equally recognizes β-arr1 and 2, a gift from Dr RJ Lefkowitz (Duke University Medical Center, Durham, NC). (**B**) Down-regulation of β-arr1 expression, by siRNA, enhances inhibition by GRK5 of the 5-HT$_4$R-activated Src/ERK pathway. HEK-293 cells expressing Myc-tagged-WT were transfected with either control or β-arr1-specific siRNAs. At 72 h after transfection, cells were stimulated 5 or 30 min before lysis. P-ERK and β-arr contents were analysed by sequential western blotting with p-ERK, and β-arr antibodies. (**C**) Quantification of ERK1/2 phosphorylation (top) and β-arr1 (bottom) was performed by densitometry using the Image J software. Data are means ± s.e.m. of results obtained in four independent experiments. $^{**}P<0.01$ versus corresponding values measured in cells transfected with control siRNA.

ERK1/2 phosphorylate β-arr1 at Ser^{412} (Lin *et al*, 1999). Thus, we hypothesized that p-S^{412}-β-arr1 rather than unphosphorylated β-arr1 mediates the inhibition, by GRK5, of 5-HT$_4$R-operated ERK signalling. We first examined whether β-arr1 associated with 5-HT$_4$R was phosphorylated at S^{412} using an antibody against p-S^{412}-β-arr1. p-S^{412}-β-arr1 co-immunoprecipitated with 5-HT$_4$R on exposure of HEK-293 cells to 5-HT (Figure 7C). The amount of p-S^{412}-β-arr1 co-immunoprecipitated with 5-HT$_4$R was largely increased in cells co-expressing GRK5 (Figure 7E). Moreover, association of p-S^{412}-β-arr1 with 5-HT$_4$R, which was transient in HEK-293 cells transfected with 5-HT$_4$R, was prolonged in cells co-expressing GRK5 and persisted for at least 10-min 5-HT stimulation (Figure 7D). Furthermore, GRK5 impaired 5-HT$_4$R internalization (Supplementary Figure S5), this observation could be relied to the presence of p-S^{412}-β-arr1 bound to the receptor as reported earlier by Luttrell *et al* (1999). Even though the magnitude of 5-HT$_4$R-induced ERK phosphorylation decreased in the presence of GRK5, we could not exclude that ERK1/2 exerted an inhibitory feedback control by phosphorylating β-arr1 at Ser^{412}. To investigate whether ERK1/2 contributed to ß-arr1 phosphorylation, we transfected MEK1(K^{97}A), a dominant negative MEK. Expression of MEK1(K^{97}A) did not affect the ratio of p-S^{412}-β-arr1/β-arr1 co-immunoprecipitated with 5-HT$_4$R (Figure 7E). Similar conclusion was obtained in cells treated with MEK inhibitors U0126 (Supplementary Figure S6) and PD 98059 (not shown).

No p-S^{412}-β-arr1 bound to the receptor was detected in cells co-transfected with GRK5 (K215R) (Figure 7C), indicating that GRK5 catalytic activity was critical for binding and/or phosphorylation of p-S^{412}-β-arr1 (Figure 7C). Inhibition, by GRK5, of 5-HT$_4$R-mediated ERK signalling in β-arr1, 2$^{-/-}$ MEF cells was only rescued by transfection of cells with β-arr1 but not with the S^{412}A-β-arr1 mutant (Figure 6). Collectively, these observations support the essential role of β-arr1 phosphorylation in the negative regulation, by GRK5, of 5-HT$_4$R-mediated ERK signalling.

GRK5 directly phosphorylates β-arrestin1

As both p-S^{412}-β-arr1 and GRK5 kinase activity were involved in the negative regulation of the Src/ERK pathway, we examined whether GRK5 directly phosphorylated β-arr1 in an *in vitro* kinase assay using purified recombinant β-arr1. We first compared the ability of GRK2 and GRK5 with phosphorylate purified β-arr1. As shown in Figure 8A, GRK5 was more efficient than GRK2 to phosphorylate β-arr1. Analysis of β-arr1 phosphorylation state by immunoblotting indicated that GRK5, but not GRK2, phosphorylated β-arr1 at S^{412} (Figure 8A). Further supporting a direct phosphorylation of S^{412} by GRK5, the GRK5-dependent ^{32}P incorporation in purified S^{412}D β-Arr1 mutant was strongly reduced, compared with that measured in WT β-Arr1 (Figure 8B). Using equivalent amounts of kinase, GRK5 was as efficient as ERK2 to promote β-Arr1 phosphorylation at S^{412} (Figure 8C).

GRK5 inhibits 5-HT$_4$R-mediated ERK signalling by preventing activation of Src constitutively associated with the receptor

Several mechanisms have been involved in activation of the Src/ERK pathway by GPCR ligands (Cao *et al*, 2000; Luttrell

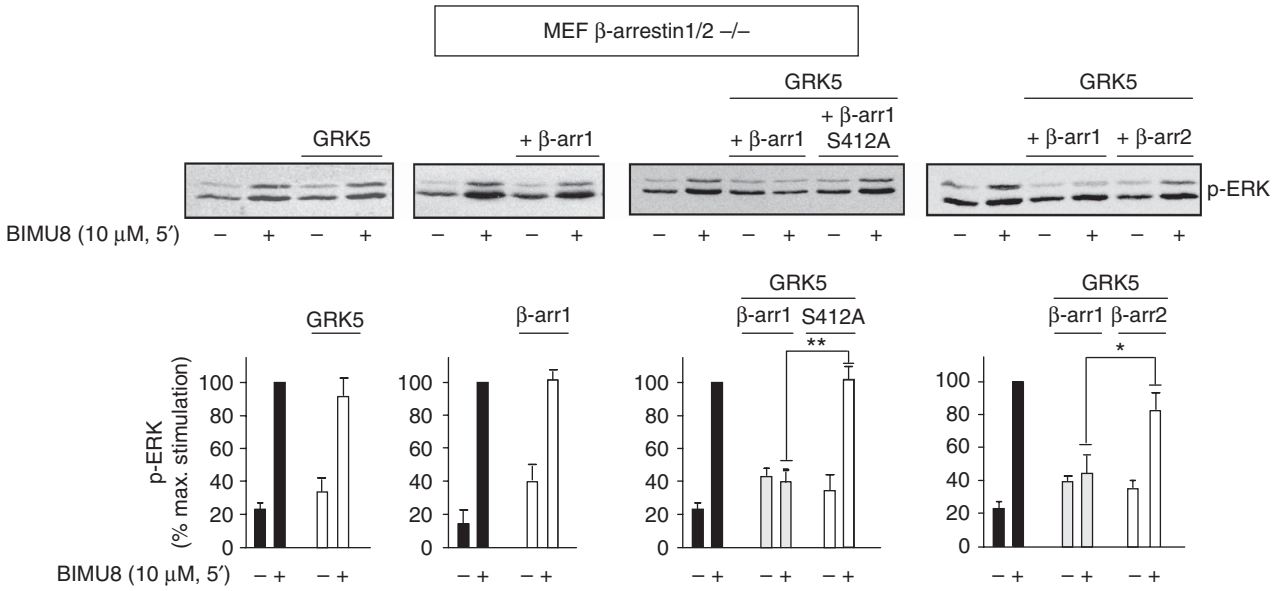

Figure 6 GRK5 fails to inhibit 5-HT$_4$R-mediated Src/ERK pathway in MEF β-arr1$^{-/-}$/ arr2$^{-/-}$. MEF β-arr1$^{-/-}$/arr2$^{-/-}$cells were transiently co-transfected with WT 5-HT$_4$R in the presence or absence of GRK5, in combination with β-arr1, β-arr1-S^{412}A mutant or β-arr2. Expression levels of the transfected WT 5-HT$_4$R construct controlled by ELISA were very similar for all the combinations. Serum starved β-arr1$^{-/-}$/arr2$^{-/-}$ MEF cells expressing the receptors were treated or not with 10 μM BIMU8 (a 5-HT$_4$ selective agonist) for 5 min. Total lysates were analysed by immunoblotting with the p-ERK1/2 antibody for all transfected MEF β-arr1$^{-/-}$/arr2$^{-/-}$cells. The histograms represented p-ERK1/2 quantified by densitometry from four western blotting, data represent mean ± s.e.m. $*P<0.05$, $**P<0.01$ versus corresponding values measured in cells transfected with both GRK5 and β-arr1 as control.

and Luttrell, 2004). For instance, activation of ERK signalling by β_2-ARs is dependent on the formation of β_2-AR/β-arr1 complex, which subsequently recruits and activates Src (Luttrell *et al*, 1999, 2001). In contrast, 5-HT$_4$Rs recruited Src independently of β-arr1. Indeed, purified Src associated with recombinant S-tagged 5-HT$_4$Rs immobilized on a S-protein agarose column (Figure 9A). Equal amounts of Src bound to 5-HT$_4$R in the absence and presence of 5-HT (Figure 9A), indicating that 5-HT$_4$R/Src interaction was direct and independent of receptor activation. Corroborating this finding, Src co-immunoprecipitated with 5-HT$_4$Rs expressed in HEK-293 cells treated or not with 5-HT (Figure 9B). Exposure of cells to 5-HT activated Src bound to the receptor, as indicated by the increased amount of p-Y^{416}-Src co-immunoprecipitated with 5-HT$_4$R (Figure 9B). Both basal and 5-HT-stimulated phosphorylations of Src associated with 5-HT$_4$Rs were strongly inhibited by GRK5 overexpression (Figure 9B). Note that phosphorylation of Src, bound to 5-HT$_4$Rs, was independent of β-arr1, as indicated by experiments performed in β-arr1/2$^{-/-}$ MEF cells (Figure 9C), and in HEK-293 cells expressing P^{91}G-P^{121}E-β-arr1, which weakly binds to Src (Luttrell *et al*, 1999) (Figure 9B). Moreover, inhibition by GRK5 of the 5-HT$_4$R-operated Src/Erk pathway was likewise independent of the interaction of Src with β-Arr, as it was not affected by expression of P^{91}G-P^{121}E-β-arr1 (Figure 9B).

Effect of GRK5 on endogenous 5-HT$_4$R-mediated ERK signalling in neurons

Finally, we examined whether the regulation of 5-HT$_4$R-mediated ERK signalling by GRK5 also occurs in neurons expressing native 5-HT$_4$Rs. Native HEK-293 cells and colliculi neurons expressed nearly similar amount of GRK5 (Figure 10A). We have shown, in HEK-293 cells, that a TAT cell-permeant Inh-Pep comprising the GRK5 binding site inhibited GRK5 binding to the receptor (Figure 4C) and thus, increased 5-HT$_4$R-stimulation of the ERK pathway (Figure 4D). Interestingly, the Inh-Pep had a similar effect on colliculi neurons: this treatment significantly enhanced ERK activation induced by maximally effective concentration of BIMU8, compared with cells treated with the control peptide ($+35\pm6\%$; $n=4$, $P<0.05$, Figure 10B). Overexpressing GRK5 in colliculi neurons clearly inhibited, as in HEK-293 cells, ERK pathway without affecting the G_s/cAMP pathway (Figure 10C). Finally, as observed in HEK-293 cells, transfection of neurons with GRK5 also attenuated 5-HT$_4$R endocytosis as shown in Supplementary Figure S5).

Discussion

Like many biological processes, the GPCR activity represents a coordinated balance between molecular mechanisms governing activation and those governing inactivation. Any deregulation of this balance may lead to pathological situations. The activation of G protein-dependent signalling by GPCRs is controlled by a well-described desensitization process, a phenomenon governed by a complex sequence of events including G protein-uncoupling, receptor phosphorylation, β-arr recruitment and receptor internalization through clathrin-coated pits (DeWire *et al*, 2007; Reiter and Lefkowitz, 2006). Recently, some GPCRs have been shown to signal through non-G protein-dependent pathways (Heuss and Gerber, 2000; Brzostowski and Kimmel, 2001) following their interaction with GIPs (GPCR interacting proteins) such as β-arrs, Src, NHERF and many others (Bockaert *et al*, 2004a, b). One of the most studied G protein-independent signalling is the ERK pathway. The most classical mechanism by which GPCRs activate ERK independently of G proteins is

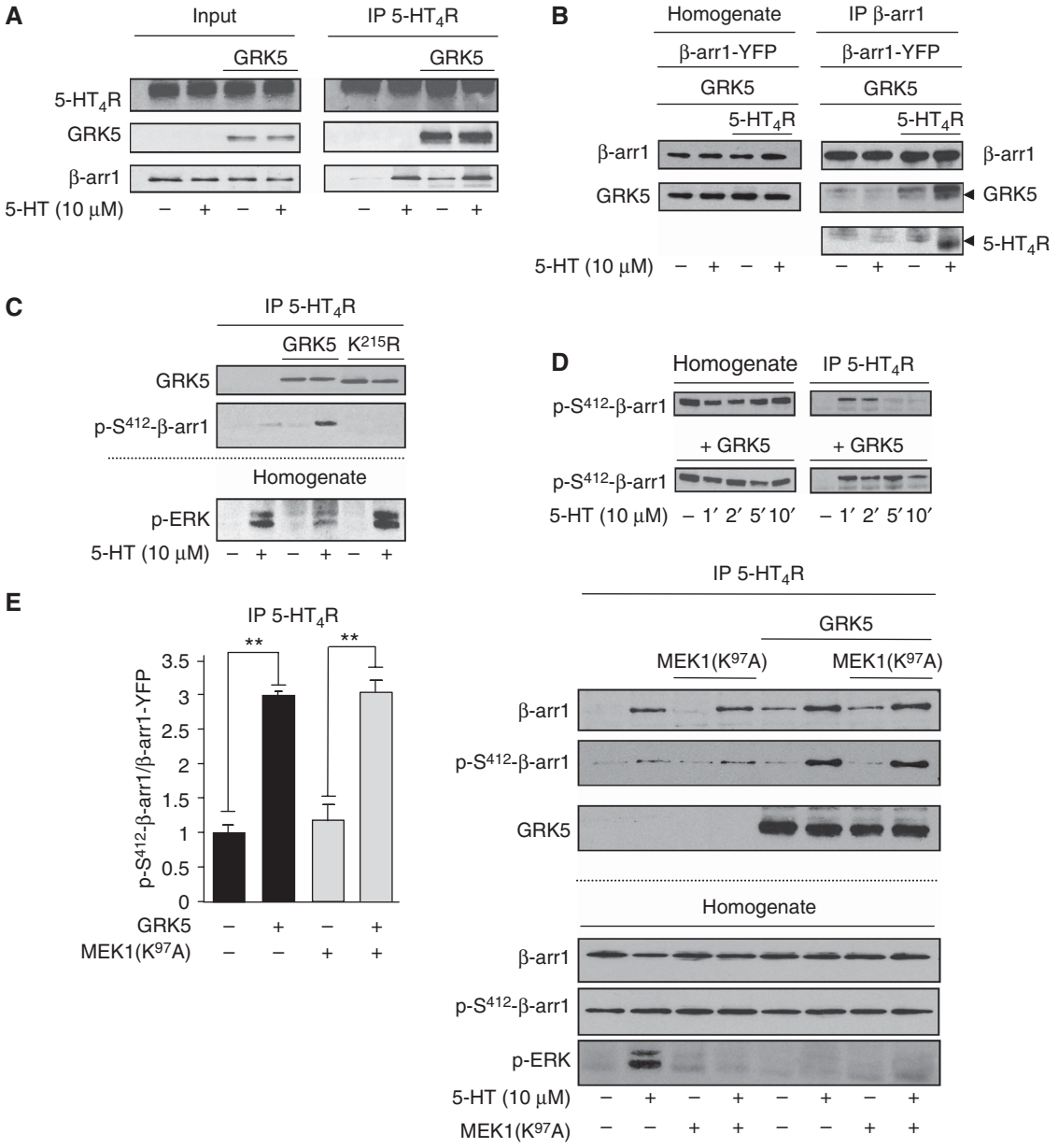

Figure 7 Inhibition, by GRK5, of 5-HT$_4$R-operated ERK signalling pathway involves p-S^{412}-β-arr1. (**A**) Cells co-transfected with Myc-tagged 5-HT$_4$R co-expressed with β-arr1-YFP and with or without GRK5 were incubated with 10 μM 5-HT for 5 min. Receptors were immunoprecipitated using anti-Myc-tagged antibody. Co-precipitated proteins (GRK5 and β-arr1) were analysed by western blotting by using the antibodies against GRK5 or against GFP for β-arr1-YFP. The immunoprecipitated receptor was revealed by using the anti-Myc antibody. On the left part, inputs represent 5% of the total protein amount used in immunoprecipitation lysates (receptor; GRK5; total β-arr1). On the right, only proteins co-immunoprecipitated with WT were visualized (GRK5, total β-arr1-YFP). (**B**) Cells co-transfected with β-arr-YFP, GRK5 with or without Myc-tagged 5-HT$_4$R were incubated with 10 μM 5-HT for 5 min. β-arr1-YFP was immunoprecipitated using anti-GFP antibody. Co-precipitated proteins (GRK5 and 5-HT$_4$R) were analysed by western blotting by using the antibodies against GRK5 or Myc-tagged. (**C**) HEK-293 cells expressing 5-HT$_4$R, β-arrestin1-YFP in the absence or the presence of GRK5 or the kinase deficient GRK5 (K^{215}R) were exposed to 10 μM 5-HT for 5 min. 5-HT$_4$R was immunoprecipitated using anti-Myc antibody. Co-precipitated proteins (GRK5, K^{215}R-GRK5, total β-arr1-YFP and phosphorylated β-arr1 (p-S^{412}-β-arr1) were analysed by western blotting. As in A, the corresponding blot of total lysate probed with antibody to p-ERK1/2 shows the correlation between the recruitment of p-S^{412}-β-arr1 and the inhibition of p-ERK. p-S^{412}-β-arr1 was revealed by using the monoclonal anti-p-S^{412}-β-arr1 antibody (Cell Signalling, Ozyme, France). (**D**) p-S^{412}-β-arr1 is rapidly dephosphorylated on receptor binding in the absence of GRK5 but is stabilized on the receptor by GRK5. HEK-293 cells transiently expressing WT 5–HT$_4$R and β-arr1-YFP in the absence or the presence of GRK5 were exposed to 5-HT 10 μM for 1, 2, 5 or 10 min. 5-HT$_4$R was immunoprecipitated as above and co-precipitated p-S^{412}-β-arr1 was analysed by western blotting. (**E**) Cells co-transfected with Myc-tagged 5-HT$_4$R, β-arr-YFP, with or without GRK5 and dominant negative of MEK1 (MEK1(K^{97}A), were incubated in the absence or presence of 10 μM 5-HT for 5 min. 5-HT$_4$R was immunoprecipitated using anti-Myc antibody. Co-precipitated proteins (total β-arr1-YFP, p-S^{412}-β-arr1 and GRK5) were analysed by western blotting. The histogram represented total β-arr1-YFP, p-S^{412}-β-arr1 quantified by densitometry from four western blotting, data represent mean ± s.e.m. $^{**}P < 0.01$ versus corresponding values measured in cells transfected in absence of GRK5 as control.

through their direct interaction with β-arrs, a process followed by receptor endocytosis and recruitment of the Src/Raf/MEK machinery (Luttrell *et al*, 1999, 2001).

We have recently shown that 5-HT$_4$Rs activate ERK through an original mechanism independently of both G proteins and β-arrs. This mechanism involves activation of

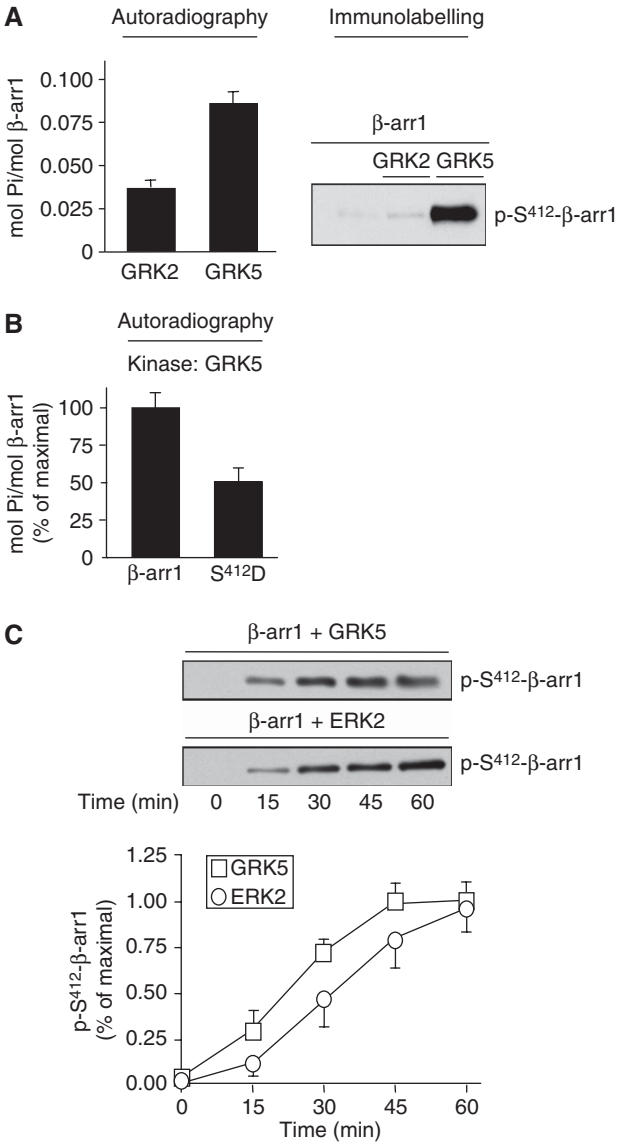

Figure 8 GRK5 phosphorylates β-arr1 *in vitro*. (**A**) Phosphorylation reactions were performed at 30 °C for 60 min with 500 nM β-arr1 and 50 nM GRK. The proteins labelled with [^{32}Pi] phosphate were analysed by SDS–PAGE and autoradiography. The stoichiometry of phosphorylation was determined by excising the arrestin bands and counting in a scintillation counter. Quantified data from three separate experiments (expressed as mean ± s.d.) are depicted. Proteins were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with an anti p-S^{412}-β-arr1 antibody. (**B**) Same experiment as in A except that phosphorylation reactions were performed with either 500 nM WT β-arr1 or 500 nM β-arr1S^{412} D mutant and 50 nM GRK5. The proteins labelled with [^{32}Pi] phosphate were analysed by SDS–PAGE and autoradiography. (**C**) Time course of β-arr1 phosphorylation in the presence of GRK5 or ERK2. Reactions were performed at 30 °C for the indicated periods (0–60 min) with 500 nM β-arr1 and 50 nM of GRK5 or 50 nM of ERK2. The western blot analysis with the anti p-S^{412}-β-arr1 antibody revealed the level of p-S^{412}-β-arr1 produced after phosphorylation with either GRK5 or ERK2. The amounts of p-S^{412}-β-arr1 were quantified by densitometry from three different experiments.

Src tightly associated with the receptor (Barthet *et al*, 2007; Figure 9). A similar mechanism has been reported for β_2-AR (Sun *et al*, 2007).

Despite accumulating data on the different routes by which GPCRs activate the ERK pathway in a G protein-independent manner, the question of their regulation is relatively unknown. Nonetheless, these studies focused on GPCRs that activate ERK through interaction with β-arrs (AT_{1A}, V_2, β_2-AR and FSH receptors) (Kim *et al*, 2005; Ren *et al*, 2005; Kara *et al*, 2006; Shenoy *et al*, 2006). Others findings reported a negative feedback regulation of ß-arrs-mediated ERK activation through a direct phosphorylation of β-arr1 by ERK (Lin *et al*, 1999; Hupfeld *et al*, 2005).

Here, we have addressed the important issue of the mechanisms involving GRK5 in the negative regulation of the 5-HT$_4$R-mediated activation of the ERK pathway. We paid particular attention to GRK5, as its recruitment to the plasma membrane is G protein-independent. GRK5 is constitutively targeted to the cell surface through the interaction with phospholipids (Kunapuli *et al*, 1994; Thiyagarajan *et al*, 2004). We found that overexpression of GRK5 potently inhibited the G protein-independent pathway, not only in HEK-293 cells but also in neurons, without significantly affecting the coupling to G_s pathway. Using purified GRK5 and 5-HT$_4$Rs, we showed that these two proteins interacted directly and that this interaction was promoted by 5-HT. In a cellular context, a constitutive interaction was observed although 5-HT increased the amount of complexes formed. Constitutive 5-HT$_4$R-GRK5 interaction may be due, at least in part, to basal 'constitutive' activity that we have described earlier (Claeysen *et al*, 1999). Indeed, the Δ346 mutant, exhibiting a higher constitutive activity than WT receptors, recruited a higher amount of GRK5 than WT receptors in the absence of 5-HT. We identified the proximal region of the 5-HT$_4$R C-t (amino-acids 330–346) as being a GRK5 binding motif. Within this region, the most important determinant for GRK5 binding was the D^{330}DE^{332} triplet. A similar result has been established by BRET for GRK5/Neurokinin-1 receptor association (Jorgensen *et al*, 2008).

We investigated the role of endogenously expressed GRK5 by using a TAT peptide, fused to the GRK5 binding motif on 5-HT$_4$R C-t (Inh-Pep). This peptide, but not a control TAT-peptide increased the ERK pathway in both HEK-293 cells and neurons, indicating that endogenous GRK5 significantly inhibited 5-HT$_4$R-operated ERK pathway. Similarly, an inhibitory role of endogenous GRK5 was also suggested by the observed increase in 5-HT$_4$R response after overexpression of the kinase dead GRK5 mutant (K^{215}R). This reveals that not only overexpressed GRK5, but also endogenously expressed GRK5 is able to inhibit 5-HT$_4$R-mediated ERK activation.

Our study also identified β-arr1 as a major player of the negative regulation of the 5-HT$_4$R-mediated Src/ERK pathway. Moreover, we demonstrate for the first time that GRK5 directly phosphorylates β-arr1 at S^{412} and that GRK5 is as potent as ERK2, the first protein kinase capable of phosphorylating β-arr1 identified (Lin *et al*, 1999). p-S^{412}-β-arr1 is the major form of cytosolic β-arr1 (Lin *et al*, 1997). On β_2-AR stimulation, the receptor interacts with p-S^{412}-β-arr1, a process followed by rapid de-phosphorylation of β-arr1. The dephosphorylated form of β-arr1 is a clathrin adaptor, and thereby allowing internalization of the β_2-AR-β-arr1 complex. In both HEK-293 and neurons, β-arr1 associated with the 5-HT$_4$R was only transiently phosphorylated (p-S^{412}-β-arr1) (1–2 min) after 5-HT exposure, a process followed by receptor internalization. In contrast, in HEK293 overexpressing GRK5, the situation was different. β-arr1 bound to the receptor remained phosphorylated up to 10 min after 5-HT exposure.

Moreover, the 5-HT$_4$R did not internalize (Supplementary Figure S5), consistent with the proposal of Lin *et al* that de-phosphorylation of β-arr1 bound to β_2-AR is necessary for its internalization (Lin *et al*, 1997).

p-S^{412}-β-arr1 was required for inhibition by GRK5 of the 5-HT$_4$R-operated activation of the ERK/Src pathway as: (1) transfection of β-arr1 in MEF β-arr1/2$^{-/-}$ rescued the GRK5-mediated inhibition and (2) transfection of the S^{412}A-β-arr1 mutant was inefficient. We then investigated how p-S^{412}-β-arr1 mediates its regulatory effect. The possibility that phosphorylation of β-arr1 known to block its interaction with Src may be involved has been excluded as we collected data showing that activation of Src, by 5-HT$_4$R, did not require β-arr1 and that overexpressing a β-arr1 mutant that inefficiently interact with Src (P^{91}G-P^{121}E-β-arr1) did not modify the capacity of GRK5 to inhibit the 5-HT$_4$R-mediated activation of the Src/ERK pathway.

The mechanisms by which Src is activated, following binding to several families of receptors, is poorly known. This is the case for β_3-ARs or even for receptor tyrosine kinases (Cao *et al*, 2000; Bromann *et al*, 2004). Many papers are 'implying' that binding of Src to the receptor is 'enough' to activate Src but 'how' is almost never described. Change in Src conformation through allosteric mechanisms is likely associated with the required de-phosphorylation of p-Tyr^{527}. In this situation, it is difficult to determine how this process can be inhibited by p-S^{412}-β-arr1. Two hypotheses can be proposed to explain the inhibitory role of p-S^{412}-β-arr1 on the inhibition of Src by 5-HT$_4$Rs. In the first one, we can propose that once phospho-β-arr is bound to the receptor, the allosteric mechanism involved in Src activation does not occur longer. In the second one, we can propose that p-S^{412}-β-arr1 affects the receptor mobility within the plasma membrane. It is likely that the 5-HT$_4$R-Src complex needs to relocate after stimulation in a domain enabling Src activation where, for example p-Tyr phosphatases are present. P-S^{412}-β-arr1 may block this relocation. This assumption appears possible as endocytosis of 5-HT$_4$Rs was totally prevented in the presence of GRK5. It is important to notice that Ser^{412} is localized within the R2 domain of β-arr1 that also contains LIEF binding motif for clathrin and the RXR binding motif for β2-adaptin (AP2) (Goodman *et al*, 1996; Laporte *et al*, 2000).

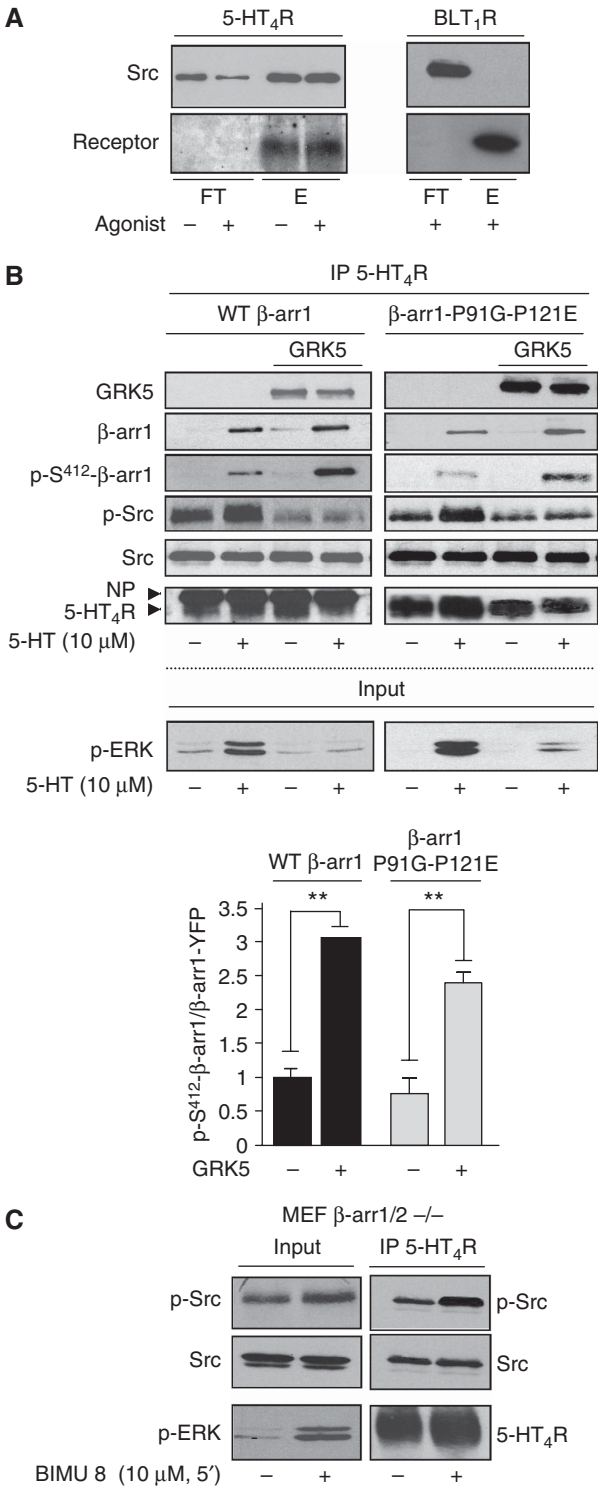

Figure 9 GRK5 affects activation of Src but not its direct interaction with 5-HT$_4$R. (**A**) Src directly interacts with the recombinant 5-HT$_4$R. The recombinant purified His-tag 5-HT$_4$R was incubated with purified full-length Src in the presence or absence of 5-HT; His-Tag BLT1, which did not interact with Src is used as control (right part). The western blot analysis with an anti-Src antibody or anti-His-tag (receptor) showed the fractions eluted from the S-agarose column. Fraction FT: or flow-through fraction contains unbound Src. Fraction E: contains Src, highly retained on the receptor and released with the receptor. – and + indicate the absence or the presence of the agonists, 5-HT for 5-HT$_4$R at 10 μM and LTB$_4$ at 10 μM for BLT1R. (**B**) Cells co-transfected with Myc-tagged 5-HT$_4$R in the presence of Flag-tagged ß-arr1 or mutant P^{91}G-P^{121}E with Src and with or without GRK5, were incubated with 10 μM 5-HT for 5 min. Immunoprecipitated 5-HT$_4$Rs as well as co-precipitated proteins (GRK5, p-Y^{416}-Src and total Src) were analysed by western blotting with the corresponding antibodies against GRK5, flag β-arr1 or mutant p-S^{412}-β-arr1, p-Y^{416}-Src, total Src and Myc for 5-HT$_4$R. The histogram represents total Flag-tagged β-arr1 versus p-S^{412}-β-arr1 co-immunoprecipitated with 5-HT$_4$Rs and total mutant P^{91}G-P^{121}E Flag-tagged β-arr1 versus p-S^{412} mutant-β-arr1 quantified by densitometry from four western blotting, data represent mean ± s.e.m. $^{**}P<0.01$ and versus corresponding values measured in cells transfected in absence of GRK5 as control. (**C**) MEF β-arr1$^{-/-}$/arr2$^{-/-}$cells were transiently transfected with WT 5-HT$_4$R, in combination. MEF β-arr1$^{-/-}$/arr2$^{-/-}$ cells expressing the receptors were treated or not with 10 μM BIMU8 (a 5-HT$_4$ selective agonist) for 5 min. Total lysates were analysed by immunoblotting with the p-ERK1/2 antibody. Immunoprecipitated 5-HT$_4$Rs as well as co-precipitated proteins (p-Y^{416}-Src and total Src) were analysed by western blotting with the corresponding antibodies againstp-Y^{416}-Src, total Src and Myc for 5-HT$_4$R.

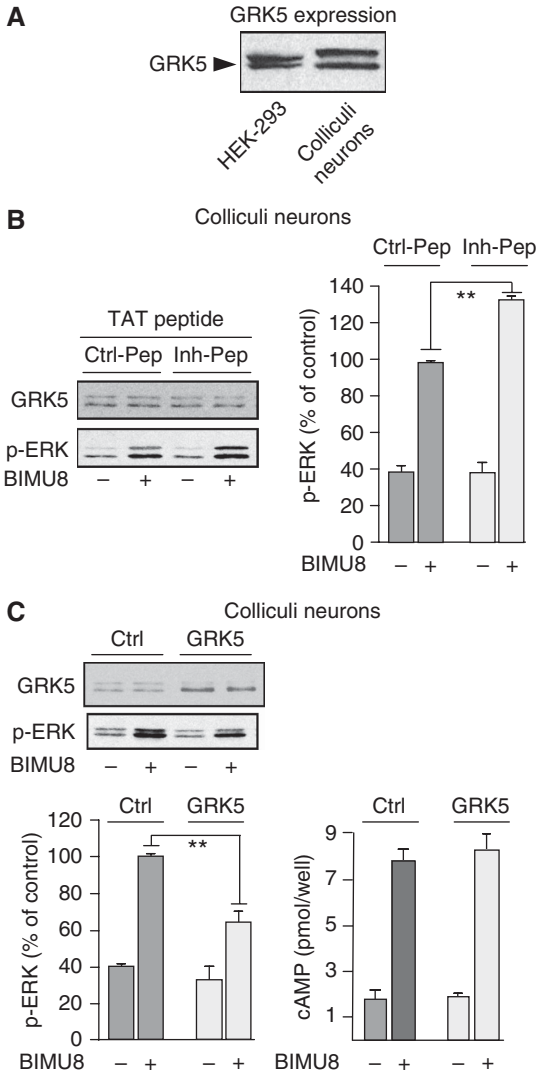

Figure 10 GRK5-mediated specific inhibition of ERK1/2 pathway also occurs in neurons. (**A**) Endogenous GRK5 expression levels in HEK-293 cells and in colliculi neurons in primary culture. (**B**) Inhibitory effect of the TAT-5-HT$_4$R C-t peptide (Inh-Pep) on GRK5-induced ERK regulation in neurons. As in Figure 4, (Inh-Pep) and TAT-peptide control (Ctrl-Pep) were pre-incubated for 1 h before the 5-min exposure to 10 μM BIMU8. Neurons were lysed in SDS sample buffer, subjected to SDS–PAGE. ERK activation (p-ERK) in the presence of the Inh-Pep was detected by western blot using p–ERK antibody. This western blotting is representative of four blots performed in the same conditions. The histogram represented the results quantified by densitometry from four western blotting, data represent mean ± s.e.m. (**C**) Colliculi neurons endogenously expressing 5-HT$_4$R were transfected with GRK5 or empty vector using lipofectamine as indicated in Materials and methods, then stimulated with vehicle or BIMU8 (a selective 5-HT$_4$R agonist). Total neuron lysates were analysed by immunoblotting with the anti GRK5 or anti p-ERK antibodies. In parallel, neurons issued from the same culture were used for cAMP production measurement. The histogram on the right shows quantification of the ERK phosphorylation and bottom histograms indicate cAMP level in neurons. Data are means ± s.e.m. of results obtained in four independent experiments. $^{**}P<0.01$ versus corresponding values measured in transfected neurons with empty vector.

Although the mechanisms by which GRK5 inhibited the 5-HT$_4$R-mediated activation of the G protein-independent Src/ERK pathway has been mainly dissected using HEK-293 cells we also demonstrated an effect of endogenous GRK5 in embryonic cultured neurons. As expression of both GRK5 and β-arr1 increases considerably during development (Gurevich and Gurevich, 2004; Gurevich *et al*, 2004), it is likely that the negative regulation of the 5-HT$_4$R-mediated activation of the G protein-independent Src/ERK pathway will be higher in adult neurons.

In conclusion, GRK5, as well as a post-translational modification of β-arr1, determine the nature of the signalling pathway (G_s or ERK) predominantly engaged on activation of 5-HT$_4$Rs. Such a control of signalling pathways could be critical for 5-HT$_4$R-dependent synaptic plasticity and memory consolidation (Kemp and Manahan-Vaughan, 2004, 2005; Marchetti *et al*, 2004; Micale *et al*, 2006).

Materials and methods

Cell cultures and transfection

HEK-293 cells and β-arr1,2$^{-/-}$ MEF cells, obtained from Professor RJ Lefkowitz (Duke University Medical Center, Durham, NC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of dialyzed foetal calf serum and antibiotics. They were transfected by electroporation, as described earlier (Claeysen *et al*, 1999). Primary cultures of colliculi neurons prepared as described earlier (Dumuis *et al*, 1988) were plated in serum-free medium in 12-well culture dishes (0.8×10^6 cells/ml, 1 ml per dish). Cultures were maintained for 8 days at 37 °C in a humidified atmosphere in 5% CO_2/95% H_2O/air and in DMEM/F12 supplemented with 10% of mixture of hormone.

Small interfering RNA transfection

The double-stranded siRNA sequence 5′-AAAGCCUUCUGCGCGGA GAAU-3′ (Eurogentec, Belgium) was used to selectively target human β-arr1 at positions 439–459 relative to the initiation codon. A small RNA duplex, which has no silencing effect, was used as a control (5′-AAGUGGACCCUGUAGAUGGCG-3′). All the siRNAs used in this study have been described earlier and validated (Ahn *et al*, 2003). A second double-stranded siRNA sequence 5′-CGC-UUA CCC-UUU-CAC-CUU-U-3 (Eurogentec, Belgium) was used to selectively target human β-arr1. A small RNA duplex that has no silencing effect was used as a control. 5′-CGU-ACG-CGG-AAU-ACU UCG-A-3′ siRNA was used to selectively target luciferase. Early passage HEK-293 cells at 40% confluence in six-well plates were transfected with 300 ng of plasmid encoding WT 5–HT$_4$R with or without 1 μg of plasmid encoding GRK5. siRNAs were transfected at 200 nM using the Gene Silencer transfection reagent according to manufacturer's recommendations (Gene Therapy Systems, San Diego). All assays were performed 3 days after siRNA transfection, as described earlier (Barthet *et al*, 2007).

Immunoblotting

Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences). Membranes were blocked for 1 h at room temperature using Tris buffered saline blocking solution containing 5% (w/v) milk powder, 0.25% v/v Tween 20. Membranes were incubated with the primary antibodies overnight at 4°C, then washed extensively, and incubated with secondary antibodies for 1 h. Immunoreactivity was detected using a Chemiluminescence Reagent Plus kit (Perkin Elmer, Courtaboeuf, France). Immunoreactive bands were quantified by densitometry using Image J and GraphPad PRISM.

Analysis of 5-HT$_4$R phosphorylation by MS

HEK-293 cells transfected with the plasmid encoding HA-tagged 5-HT$_4$R and/or GRK constructs were grown in 100-mm culture dishes (five dishes containing 10×10^6 cells per condition). Cells were lysed in 5 ml solubilization buffer (Hepes 20 mM, 150 mM NaCl, 1% NP40, 10% glycerol, 4 mg/ml dodecylmaltoside supplemented with protease inhibitors and phosphatase inhibitors (NaF, 10 mM; Na^+-vanadate, 2 mM; Na^+-pyrophosphate, 1 mM and β-glycerophosphate, 50 mM). Receptors were immunoprecipitated on anti-HA agarose beads (Sigma Aldrich). Immunoprecipitated proteins were resolved by SDS–PAGE. After reduction and alkylation (with 10 mM DTT for 45 min at 56°C and 55 mM

iodoacetamide for 30 min at room temperature), receptors were digested in-gel by trypsin (600 ng, Gold, Promega) and trypsic peptides were extracted from the polyacrylamide, as described earlier (Shevchenko *et al*, 1996). Trypsic digests were enriched in phosphopeptides using the PHOS-Select Iron Affinity Gel (Sigma Aldrich), as described by the manufacturer, dehydrated in a vacuum centrifuge, and solubilized in 3 µl of 0.1% formic acid, 2% acetonitrile in water. Samples were analysed online by nano-flow HPLC-nanoelectrospray ionization using a LTQ Orbitrap mass spectrometer (LTQ Orbitrap XL, Thermofisher Scientific) coupled with an Ultimate 3000 HPLC (Dionex). Desalting and pre-concentration of samples were performed on-line on a Pepmap® precolumn (0.3 × 10 mm). A gradient consisting of 0–40% A in 30 min, 40–80% B in 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nl/min was used to elute peptides from the capillary (0.075 × 150 mm) reverse-phase column (Pepmap®, Dionex). LC-MS/MS experiments comprised cycles of six events: a MS^1 scan with Orbitrap mass analysis at 15 000 resolution followed by HCD (higher energy collisional dissociation) of the five most abundant precursors. Fragment ions generated by HCD were detected in the Orbitrap analyzer at 7500 resolution. Normalized collision energy of 45 and activation time of 30 ms were used for HCD. All Spectra were recorded under positive ion mode using the Xcalibur 2.0.7 software (Thermofisher Scientific). The mass scanning range was m/z 400–2000 and the capillary temperature was 200°C. Source parameters were adjusted as follows: Ion spray voltage, 2.45 kV; capillary voltage, 20 V and tube lens, 140 V. Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient. Spectra were analysed using Proteome Discover software v 1.0 (Thermofisher Scientific). MS/MS spectra were searched against the sequence of the HA-tagged 5-HT$_{4a}$R by using the Mascot v 2.2 algorithm (http://www.matrixscience.com). All spectra for phosphorylated peptides were validated by visual inspection.

β-*arrestin phosphorylation assay*

Phosphorylation of β-arr were performed using 500 nM purified β-arr1 or β-arr2 (Milano *et al*, 2002) and 50 nM purified GRK2 or GRK5 (Kim *et al*, 1993; Kunapuli *et al*, 1994), in the presence or absence of 0.1 mg/ml crude soybean phosphatidylcholine. The phosphorylation medium (20 µl) including 100 µM [γ-^{32}P]ATP (2.0–7.5 cpm/fmol), 20 mM Tris–HCl, pH 7.5, 2 mM EDTA, and 7.5 mM $MgCl_2$ for 60 min at 30 °C. SDS sample buffer was added to terminate the reaction and proteins were resolved by SDS–PAGE (10%). After autoradiography, the ^{32}P incorporation into β-arr was determined by Cerenkov counting.

To determine the site of phosphorylation, the above reaction was performed in the absence of radiolabelled ATP. Reaction mixtures were also subjected to western blotting to determine β-arr1 phosphorylation on S412 by using the p-S^{412}-β-arr1 antibody.

Co-immunoprecipitation

Cells were transfected with WT or truncated and mutated Myc-tagged-5-HT$_4$R constructs generated as described earlier (Claeysen *et al*, 1999). Cells were seeded in 150 mm plate (10^6 cells by plate) 48 h before the experiment. They were exposed for 5-min to 10 µM 5-HT stimulation with 10 µM at 37°C in DMEM without serum. Then, the cross-linking reaction was realized during 30 min in PBS completed with 1.25 mM of dithiobissuccinimidyl propionate (Pierce, Perbio-Brebières, France) a membrane–permeable, hydrolysable, covalent cross-linker. The cross-linking reaction was stopped with Tris 50 mM pH 7,4. After two washes in PBS, cells were incubated solubilization buffer (see above). For 30 min at 4°C, samples were centrifuged at 15 000 r.p.m. for 15 min. Solubilized proteins were incubated overnight at 4 °C with 20 µl of protein A/G-sepharose beads pre-coupled with 8 µg of antibody. Immunoprecipitated proteins were eluted in Laemmli sample buffer, resolved by SDS–PAGE and detected by western blotting.

In vitro protein interaction assay

Recombinant 5-HT$_4$R was produced and purified as described earlier (Baneres *et al*, 2005), which includes an S-tag at its N-terminus and an His-Tag $(His)_6$ sequence at its C-terminus. Purified receptors were incubated with either purified GRK5 (Cell signalling Technology) or purified human Src (Sigma) in the absence or presence 10 µM 5-HT. Protein concentrations in the 5 µM range were used. After 1 h incubation at 15 °C, the complex was loaded on a S-protein agarose column (100 µl resin) and the flow-through fraction recovered (FT fractions). The column was washed with a first buffer containing 12.5 mM sodium borate pH 7.8, 25 mM KCl, 1% DMPC, 1% CHAPS, and 0.02% cholesteryl hemisuccinate. Finally, the receptor immobilized on the column and associated proteins were recovered with a 12.5 mM sodium borate, 25 mM KCl, pH 7.8, 1% DMPC, 1% CHAPS, 0.02% cholesteryl hemisuccinate, 3 M $MgCl_2$ buffer (E fractions).

Data analysis

All data represented correspond to the means ± s.e.m. of at least three independent experiments performed in triplicate. Statistical analysis was carried out with the *t*-test using GraphPad Prism 3.0 software. *P*-values <0.05 were considered as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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