Figures and figure supplements

Phosphorylation of β-arrestin2 at Thr$^{383}$ by MEK underlies β-arrestin-dependent activation of Erk1/2 by GPCRs

Elisabeth Cassier et al
Figure 1. 5-HT$_{2C}$ and 5-HT$_{4}$ receptor stimulation promotes β-arrestin2 phosphorylation in HEK-293 cells. (A) Ribbon diagram of rat β-arrestin2 showing the position of phosphorylated residues identified by LS-MS/MS. (B) Representative extracted ion chromatograms of the EIDIPVDNTLEFDTNYAP$^{383}$TDDDIVFEDFAR peptide from YFP-tagged β-arrestin2 in cells expressing 5-HT$_{2C}$ or 5-HT$_{4}$ receptor and challenged with vehicle (Basal) or 5-HT (1 and 10 μM, respectively) for 5 or 30 min. Two other independent experiments performed on different sets of cultured cells yielded similar results. The histogram represents the means ± SEM of ion signal intensities of the peptide obtained in the three experiments. (C) 5-HT$_{2C}$ or 5-HT$_{4}$ receptor expressing cells were treated as in (B). Erk1,2 activation and Thr$^{383}$ phosphorylation were assessed by Western blotting using the anti-phospho-Thr$^{202}$/Tyr$^{204}$-Erk1/2 and the anti-phospho-Thr$^{383}$ β-arrestin2 antibody, respectively. The histogram shows the means ± SEM of the anti-phospho-Thr$^{383}$ antibody.

Figure 1 continued on next page
Figure 1 continued

phospho-Thr$^{383}$ β-arrestin2 immunoreactive signals (expressed in arbitrary unit) obtained in three independent experiments performed on different sets of cultured cells. One-way ANOVA: (B) F(5,12)=7.544, p=0.0020; (C) F(4,10) = 4.417, p=0.0259. *p<0.05 vs. corresponding vehicle.

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The following source data is available for figure 1:

**Source data 1.** List of phosphorylated peptides identified from purified β-arrestin1 and β-arrestin2 by nano-LC-MS/MS.

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**Source data 2.** This file contains raw values used to build Figure 1C.

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Figure 1—figure supplement 1. Purification of YFP-β-arrestin1 and YFP-β-arrestin2 co-expressed with 5-HT_{2C} or 5-HT_{4} receptor in HEK-293 cells. YFP-β-arrestin1 (A) and YFP-β-arrestin2 (B) co-expressed with Myc-tagged 5-HT_{2C} or 5-HT_{4} receptor were immunoprecipitated using GFP Trap beads and detected by Western blotting using an anti-GFP antibody (10% of IP) and by colloidal Coomassie blue staining (90% of IP). Receptor expression and functionality were assessed by immunoblotting using an anti-Myc antibody, and by sequential immunoblotting with the antibody recognizing phospho-Thr^{202}/Tyr^{204}-Erk1/2 and total Erk1/2. Immunoblots and gels representative of four independent experiments are illustrated. Note that 5-HT_{4} receptor immunoreactivity was detected at molecular weights corresponding to receptor monomer and dimer.

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**A** β-arrestin 1 coverage: 88% (exp pvalue cut off 0.01)

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1  MGDKCTRVK KASPNGKLT VLYGKRDFVDH  IDLVDPVDVG VLVDPEYLKE
51  RRVVYTLTCA FRYGRRLVD LGTLFRKDLF  VANVQSFPPA PEEKFLTRL
101 QRLKIKLGE HAYIFFFPE PNLPCSVTLP  PGDPTGKAC GDYEVKAF
151 AENLEKILK NSVRLVIRK VQYAPERPG  OQTAETTRQP LMSDKFLHLE
201 ASLDKEVYGY EPI3SVNHV TNNNTKTVKK  IKS3VQRQYAD ICLFNTAQYK
251 CFVAMMEADD TVAPSSTFCY VYTLTPFLAN  NREKRLGLD GKLKHDNTNL
301 ASSTLRLREGA NRELGIIVS YKVVKKVLS  RGL誓CDLAS S3DI3VELFPT
351 LMHPKPKGEP PHREVPESET PVD74NLEILD TND3DIVFED PQRQRLGKMK
401 DDKDEEEDGTV SP3HLNRR
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**B** β-arrestin 2 coverage: 85% (exp pvalue cut off 0.01)

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1  MGEPGPRVF KKS3PCLLT VLYGKRDFVD  HLDKVPFDVG VV3DPEYLK
51  DKRFLVTLTCA AF1YGRRLDL LGVLFRKDLF  IFATYQAFPP MNPARPPTTR
101 LOQSSLKLKG OAHHPFFFTI PQLPCSVTLP  QPGEDFGLA C3VDPEIRAF
151 C0K3EEKSH KRSN3VLIIR KVQFAPET178PG PQ6PS3ETTH  FL7MS194D3RS199LH
201 LEASDKEKLY YEGP3PLNVY HVT3N3AKTV  KKR3VSRRQY ADICLF3STQA
251 YKCPVAVLEQ DD3VSP3S267S268TF C4KVTITPLL  S281D3N3RKGLA LDGQLKHEDT
301 NLAS3TIVE GAN3KEVLIL VSYR3KV3KLV  VSR3GDS3VSE L3P3MVLPK
351 HDH3ITPRQ P343AP3REIDIPV D3TN3L3BDFDTN  YAT383D3D3IV3EF DC3ARL3RLGKGM
401 KD3DCD3Q3FC
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**Figure 1—figure supplement 2.** Sequence coverage of β-arrestin1 and β-arrestin2 obtained by LC-MS/MS. The sequence covered by LC-MS/MS analysis is highlighted in red. Identified phosphorylated residues in β-arrestin1 (Thr\(^{374}\)) and β-arrestin2 (Thr\(^{178}\), Ser\(^{194}\), Ser\(^{267/268}\), Ser\(^{281}\), Ser\(^{361}\) and Thr\(^{383}\)) and their positions are highlighted in blue.

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Figure 1—figure supplement 3. Tandem mass spectra of EVPESETPVD<sub>374</sub>NLIELDTNDDDIVFEDFAR, CPVAQLEQDDQVSP<sub>S<sub>267</sub>S<sub>268</sub></sub>TFCK and EIDIPVDTNLIEFD TNYApT<sub>383</sub>DDDIVFEDFAR phosphorylated peptides identified from YFP-tagged β-arrestin1 and β-arrestin2 transiently co-expressed.
with 5-HT$_{2C}$ receptor in HEK-293 cells and immunoprecipitated using the GFP Trap kit. For each identified phosphorylated peptide, MS/MS spectra that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length protein are illustrated.

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Figure 1—figure supplement 4. Tandem mass spectra of HFLMpS194DRR, KVQFAPE pT178PGPQPSAETTR and PHDHITLPRPQpS361APR phosphorylated peptides identified from YFP-tagged β-arrestin1 and β-arrestin2 transiently co-expressed with 5-HT2C receptor in HEK-293 cells and Figure 1—figure supplement 4 continued on next page.
immunoprecipitated using the GFP Trap kit. For each identified phosphorylated peptide, MS/MS spectra that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length protein are illustrated.

DOI: 10.7554/eLife.23777.008
Figure 1—figure supplement 5. Tandem mass spectra of VQFAPEpT$^{178}$PGPQPSAET TR, VYTITPLL$^{281}$SDNR, VYTITPLLpS$^{281}$DNREK phosphorylated peptides identified from YFP-tagged β-arrestin1 and β-arrestin2 transiently co-expressed with 5-HT$_{2C}$ receptor in HEK-293 cells and

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immunoprecipitated using the GFP Trap kit. For each identified phosphorylated peptide, MS/MS spectra that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length protein are illustrated.

DOI: 10.7554/eLife.23777.009
**A**

Unfolding of β-arrestin C-tail

**B**

HEK-293 cells

**C**

In vitro kinase assay
Figure 2. Role of MEK in the phosphorylation of β-arrestin2 at Thr^{383} elicited by 5-HT_{2C} receptor stimulation. (A) Mechanistic model of assembly of the 5-HT_{2C} receptor/β-arrestin2/Erk module. Color code: receptor in orange, MEK in green, β-arrestin2 core in pale cyan and C-tail in cyan (the regions 351–384 and 394–419, which are not visible in 3D structure are represented by dashed lines, the region 385–393 is represented by spheres), Erk in dark red, Raf-1 RBD domain in pink. In this model, we hypothesize that Thr^{383} phosphorylation by MEK takes place within the assembled receptor/β-arrestin2/Raf/MEK complex and results in a movement of β-arrestin2 unfolded 350–393 segment away from the first β-strand of β-arrestin, leaving space for further interaction with the receptor C-terminal domain (orange spheres) and recruitment of Erk, and its subsequent phosphorylation by MEK. For the clarity of the figure, the extremity of the β-arrestin C-tail is represented by spheres even in its unfolded state, although the real 3D structure is unknown. (B) Representative extracted ion chromatograms of the peptide in cells expressing 5-HT_{2C} receptor, pretreated with either vehicle (control) or FR180204 (10 µM for 18 hr) or U0126 (5 µM for 30 min) or coexpressing MEK1 dominant-negative mutant (MEK1DN), and challenged with vehicle (Basal) or 5-HT (1 µM) for 30 min. The histogram represents the means ± SEM of the corresponding ion signal intensities (normalized to values in 5-HT-stimulated cells in Control condition) obtained in three independent experiments. One-way ANOVA: F(8,18) = 15.69, p<0.0001. ***p<0.001 vs. corresponding basal value. (C) YFP-tagged β-arrestin2 (wild-type or Thr^{383}Ala mutant) purified from transfected HEK-293 cells was incubated with active MEK1 for 15 min at 37°C. When indicated, U0126 (5 µM) was included in the incubation medium. Thr^{383} phosphorylation was assessed by sequential immunoblotting with the antibody raised against phospho-Thr^{383} β-arrestin2 and the anti-β-arrestin2 antibody. Means ± SEM of results from four independent experiments are shown on the histogram. n.d.: not detectable. One-way ANOVA: F(2,9) = 352.2, p<0.0001. ***p<0.001 vs. immunoreactive signal in absence of MEK; §§§ p<0.001 vs. corresponding condition in absence of U0126.

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The following source data is available for figure 2:

Source data 1. This file contains raw values used to build Figure 2B, C.

DOI: 10.7554/eLife.23777.011
Figure 2—figure supplement 1. Thr$^{383}$ phosphorylation is not mediated by casein kinase 2. (A) Representative extracted ion chromatograms of the EIDIPVDNLIEFDTNYAp$^{383}$TDDIVFEDFAR peptide from YFP-tagged β-arrestin2 in cells expressing 5-HT$_{2C}$ receptor and challenged with vehicle (Basal) or 1 μM 5-HT for 30 min in absence or presence of TBCA (1 μM, added 15 min before the onset of 5-HT treatment). Two other independent experiments performed on different sets of cultured cells yielded similar results. (B) The histogram represents the means ± SEM of ion signal intensities of the peptide (normalized to values in 5-HT-stimulated cells) obtained in the three experiments. One-way ANOVA F(3,8) = 6.762, p=0.013. *p<0.05 vs. corresponding vehicle.

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The following source data is available for figure 2:

Figure supplement 1—Source data 1. This file contains raw values used to build Figure 2—figure supplement 1.

DOI: 10.7554/eLife.23777.013
Figure 2—figure supplement 2. Impact of MEK and Erk1/2 pharmacological inhibitors and of co-expression of MEK dominant-negative mutant on 5-HT$_{2C}$ receptor-operated Erk1/2 and Elk1 phosphorylation. (A) Cells expressing 5-HT$_{2C}$ receptor were pretreated with vehicle or U0126 (5 μM for 30 min) or FR180204 (10 μM for 18 hr) and then exposed to vehicle or 5-HT (1 μM) for 5 min. When indicated, a dominant-negative mutant of MEK (MEKDN) was co-expressed by 5-HT$_{2C}$ receptor. Erk1/2 activation was assessed by sequential immunoblotting with the antibody recognizing phospho-Thr$^{202}$/Tyr$^{204}$-Erk1/2 and total Erk1/2. Immunoblots representative of three independent experiments are illustrated. Note that FR180184 did not affect 5-HT$_{2C}$ receptor-operated Erk1/2 phosphorylation, indicating that it does not inhibit MEK. (B) The impact of MEK and Erk1/2 inhibitors on phosphorylation of the Erk1/2 substrate Elk1 was assessed by sequential immunoblotting with the antibody recognizing phospho-Elk1 and total Elk1. Immunoblots representative of two independent experiments are illustrated.

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In vitro phosphorylation of β-arrestin2 versus Erk2 by MEK1. YFP-tagged β-arrestin2 (wild-type or Thr\(^{383}\)Ala mutant) purified from transfected HEK-293 cells or purified non-activated Erk2 (∼1 µg each) were incubated with active MEK1 in presence of \([γ-\text{\textsuperscript{32}P}]\)-ATP (2 µCi/nmol) for 10 min at 37°C. Proteins were separated by SDS-PAGE and stained with Coomassie colloidal blue (top image) and \(\text{\textsuperscript{32}P}\) incorporation into the different substrates was monitored by autoradiography (bottom image). The data in the histogram, expressed in nmol/min/mg enzyme, represent the means ± SD of \(\text{\textsuperscript{32}P}\) incorporation into β-arrestin2 and Erk2 protein bands in the corresponding experiment after radioactive background subtraction for each lane.

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The following source data is available for figure 2:

**Figure supplement 3—Source data 1.** This file contains raw values used to build *Figure 2—figure supplement 3*.

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Figure 3. Thr383 phosphorylation underlies β-arrestin2 conformational rearrangement elicited by 5-HT2c receptor stimulation. (A, B) Translocation of wild type (WT), T383A and T383D Rluc-β-arrestin2 to Myc-5-HT2c-YFP (A) or Myc-5-HT4-YFP (B) receptors in cells treated with either vehicle (Basal) or 1 or 10 μM 5-HT, respectively, was measured by BRET. Data represent the mean ± SEM of values obtained in three independent experiments and were Figure 3 continued on next page.
Figure 3 continued

normalized to the BRET signals measured in 5-HT-stimulated cells expressing WT Rluc β-arrestin2. (C, D) Cell surface expression of receptors was measured in the same experimental condition by ELISA using anti-Myc antibody. Data are the mean ± SEM of values obtained in three independent experiments. They were normalized to total receptor expression level and are expressed in % of basal receptor level at the cell surface in cells expressing WT β-arrestin2. (E, F) Conformational arrangement of WT, T383A and T383D double brilliance Rluc8-β-arrestin2-RGFP elicited by 5-HT2C and 5-HT4 receptor stimulation by 5-HT (1 and 10 μM, respectively). Equivalent expression of each BRET sensor was verified by ELISA. Data represent the mean ± SEM of values obtained in three independent experiments and were normalized to the basal intra-molecular BRET signal in cells expressing WT Rluc8-β-arrestin2-RGFP. One-way ANOVA: A, F(5,12)=10.75, p=0.0004; B, F(5,12)=320.9, p<0.001; C, F(6,14)=10.82, p<0.001; D, F(6,14)=48.52, p<0.0001; E, F(5,12)=5.136, p=0.0095; F, F(5,12)=6.436, p=0.004. *p<0.05, **p<0.01 ***p<0.001 vs. corresponding basal.
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The following source data is available for figure 3:

Source data 1. This file contains raw values used to build Figure 3.
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Figure 4. Phosphorylation of β-arrestin2 at Thr^{383} is a necessary step in Erk1/2 recruitment to β-arrestin2 and engagement of Erk signaling by 5-HT_{2C}. Figure 4 continued on next page.
receptor. (A, B) Recruitment of Erk1/2 to WT, T^{383}A and T^{383}D YFP-β-arrestin2 in cells expressing 5-HT\textsubscript{2C} or 5-HT\textsubscript{4} receptor and exposed or not to 5-HT (1 and 10 μM, respectively) was assessed by co-immunoprecipitation. Immunoblots representative of three independent experiments are illustrated. The histograms represent the means ± SEM of Erk1/2 immunoreactive signals in immunoprecipitates, assessed by densitometric analysis, obtained in the three experiments. They were normalized to the amount of YFP-β-arrestin2 immunoprecipitates and expressed in % of basal level measured in cells expressing WT β-arrestin2. *p<0.05 vs. basal value in cells expressing WT β-arrestin2. (C, D) Erk1/2 activation in cells co-expressing 5-HT\textsubscript{2C} or 5-HT\textsubscript{4} receptor and either WT, or T^{383}A and or T^{383}D YFP-β-arrestin2 and exposed or not to 5-HT (1 and 10 μM, respectively) for 5 or 30 min was assessed by sequential immunoblotting with the antibody recognizing phospho-Thr\textsuperscript{202}/Tyr\textsuperscript{204}-Erk1/2 and total Erk1/2. Immunoblots representative of three independent experiments are illustrated. The histograms represent the means ± SEM of values (normalized to the level of phosphorylated Erk1/2 in cells expressing WT β-arrestin2 and exposed to 5-HT for 5 min) obtained in the three experiments. One-way ANOVA: A, F(5,12)=4.305, p=0.00178; B, F(5,12)=0.977, p=0.47; C, F(8,18)=11.78, p<0.001; D, F(8,18)=4.998, p=0.0022. *p<0.05, **p<0.01 vs. corresponding value in cells expressing WT β-arrestin2.

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The following source data is available for figure 4:

Source data 1. This file contains raw values used to build Figure 4.

DOI: 10.7554/eLife.23777.020
Figure 4—figure supplement 1. Role of MEK in Erk recruitment to β-arrestin2. HEK-293 cells co-expressing YFP-β-arrestin2 and 5-HT$_{2C}$ receptor were treated for 30 min with vehicle or 1 μM 5-HT in absence or presence of 5 μM U0126 (added 15 min before the onset of 5-HT application). Recruitment of Erk1/2 to β-arrestin2 was assessed by co-immunoprecipitation. Immunoblots representative of three independent experiments are illustrated. The histogram represents the means ± SEM of Erk1/2 immunoreactive signals in immunoprecipitates normalized to the amount of YFP-β-arrestin2 immunoprecipitates, obtained in the three experiments. They were expressed in % of Erk immunoreactive signal measured in 5-HT-stimulated cells. One-way ANOVA: F(4,10) = 12.21, p=0.0007. **p<0.01 vs. vehicle.

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The following source data is available for figure 4:

Figure supplement 1—Source data 1. This file contains raw values used to build Figure 4—figure supplement 1.

DOI: 10.7554/eLife.23777.022
Figure 5. Phosphorylation of β-arrestin2 at Thr383 underlies β-arrestin-dependent engagement of Erk1/2 signaling by FSH, β-adrenergic and CXCR4 receptors. Erk1/2 activation elicited by stimulation for the indicated times of transiently expressed FSH receptor (FSH, 3.3 nM), native β-adrenergic (isoproterenol 1 μM) and CXCR4 receptors (CXCL12, 10 nM) in cells transfected with control siRNA or β-arrestin2 siRNA (A–C) and in cells coexpressing WT or T383A β-arrestin2 (D–F). Erk1/2 activation was assessed by sequential immunoblotting with the antibody recognizing phospho-Thr202/Tyr204-Erk1/2 and total Erk1/2. Immunoblots representative of three independent experiments are illustrated. The histograms represent the means ± SEM of values (normalized to the level of phosphorylated Erk1/2 in cells exposed to agonist for 5 min) obtained in the three experiments. One-way ANOVA: A, F(5,12) =8.178, p=0.0014; B, F(5,12)=16.97, p<0.001; C, F(5,12)=20.22, p<0.001; D, F(5,12)=7.710, p=0.0019; E, F(5,12)=29.76, p<0.0001; F(5,12)=8.695, p=0.0012; *p<0.05 and **p<0.01 vs. corresponding value in control siRNA transfected cells or cells expressing WT β-arrestin2.

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The following source data is available for figure 5:

Source data 1. This file contains raw values used to build Figure 5.
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Figure 5—figure supplement 1. Down-regulation of β-arrestin2 protein expression in HEK-293 cells using siRNA. Cells were transfected with either control or β-arrestin2 siRNA in absence (Mock, A) or presence (B) of the plasmid encoding FSH receptor. Cells were lysed 72 hr after transfection and expression of β-arrestin2 was measured by Western blotting. Equal loading was assessed by immunoblotting using a GAPDH antibody. Representative immunoblots of three independent experiments are illustrated. The histograms show the quantification of β-arrestin2 immunoreactivity in the corresponding experiments. Data, expressed in % of value measured in cells transfected with control siRNA, are means ± SEM of results obtained in three independent experiments. Unpaired t-test, A, \( p=0.008 \) vs. control siRNA; B, \( p=0.0017 \) vs. control siRNA.

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The following source data is available for figure 5:

Figure supplement 1—Source data 1. This file contains raw values used to build Figure 5—figure supplement 1. DOI: 10.7554/eLife.23777.026
Figure 6. Schematic representation of the sequence of events proposed for β-arrestin-dependent Erk1/2 activation by GPCRs. β-arrestin, Raf and MEK are recruited to agonist-stimulated receptor, resulting in MEK activation. Active MEK phosphorylates β-arrestin2 at Thr<sup>383</sup>. This induces a movement of the β-arrestin2 350–393 segment away from the first β-strand of β-arrestin, leaving space for its interaction with the C-terminal domain of the receptor. Erk then binds to the complex and is activated by MEK. The dash circles represent activated enzymes.

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