SUPPLEMENTARY DATA

EXPERIMENTAL PROCEDURES

Co-immunoprecipitation assay

SH-NPFF$_2$-YFP cells (expressing hNPFF$_2$ receptor fused to Yellow Fluorescent Protein (YFP) at the cytoplasmic tail) were grown in 100 mm dishes. On the day of experiment, the culture medium was removed and the cells washed with 5 ml of KRH buffer. The cells were then incubated for 2 min at room temperature with 5 ml of KRH buffer containing, or not, 1 µM 1DMe, followed by cross-linking for 30 min in PBS containing 1 mM DSP (dithiobis[succimidylpropionate], ThermoFisher Scientific, France). After quenching with 5 ml of Tris 10 mM pH 7.4, cells were harvested and centrifuged at 1000 × *g* for 15 min at 4 °C. The pellet was resuspended in 0.5 ml of freshly prepared immunoprecipitation buffer consisting in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% NP40 (Nonidet) and protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science, France) and processed for immunoprecipitation as described in the experimental procedure section of the manuscript. The immunoprecipitate was solubilized in Laemmli sample buffer, denatured at 100 °C and resolved on 10% polyacrylamide gel.

FIGURE LEGENDS

Suppl. Fig. 1. A, Quantification of the total amount of MOP-YFP receptors by anti-GFP immunoblotting. An equivalent number of (SH$_2$-D9)MOP-YFP cells were treated for 30 min at room temperature with buffer alone, 1µM DAMGO, 1µM DAMGO + 1 µM 1DMe, 1 µM 1DMe or 10 µM clonidine and harvested in Laemmli loading buffer. After detection of pS377, membranes were stripped and re-probed with an anti-GFP antibody to quantify total amounts of receptors. Histogram represents means ± S.E. of band quantification from at least 3 independent experiments performed in duplicate. One-way ANOVA revealed no statistical effect of any treatment.

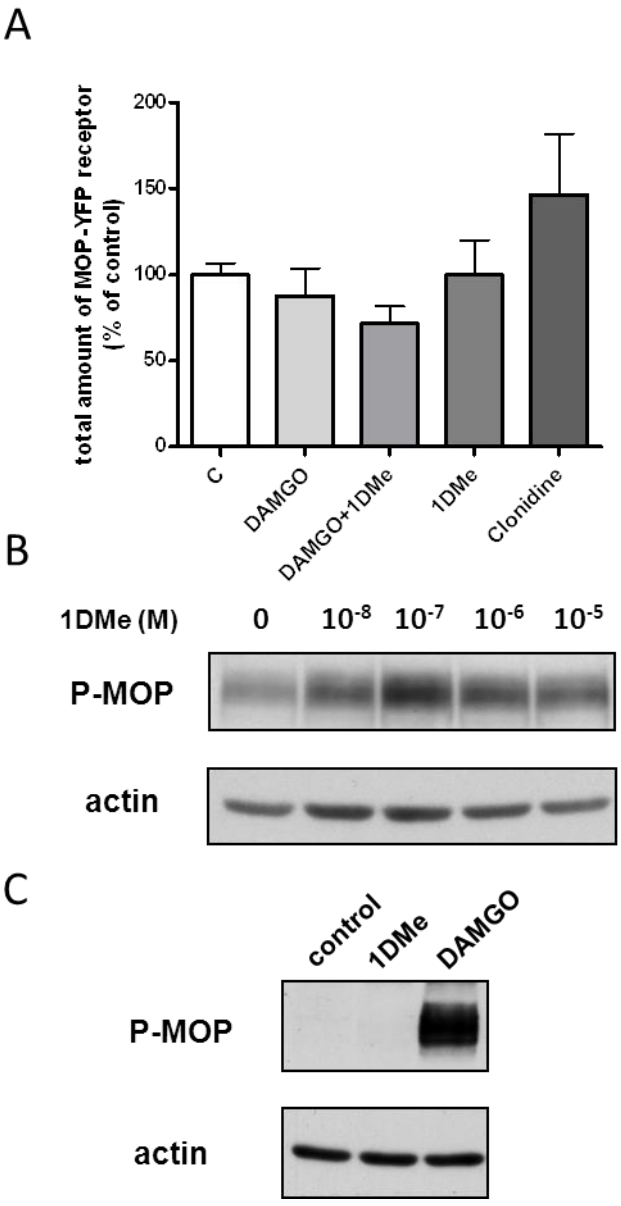
B, Effect of increasing doses of 1 DMe on S377 phosphorylation of MOP receptor. An equivalent number of cells were treated for 30 min at room temperature with buffer alone or increasing doses of 1DMe, and harvested in loading buffer. Samples were immunoblotted with anti-pS377 antibody (P-MOP) followed by anti-actin antibody for normalization. The figure shows a representative result from 3 independent experiments.

C, No effect of 1DMe on S377 phosphorylation of MOP receptor in cells that do not express $NPFF_2$ receptors. SH-SY5Y cells stably expressing T7 tagged human MOP receptors were treated for 30 min at room temperature with buffer alone or 1 µM of 1DMe or DAMGO, and harvested in loading buffer. Samples were immunoblotted with anti-pS377 antibody followed by anti-actin antibody for normalization. The P-MOP band migrated between the 75 and 95 kDa molecular weight markers. The figure shows a representative result from 3 independent experiments.

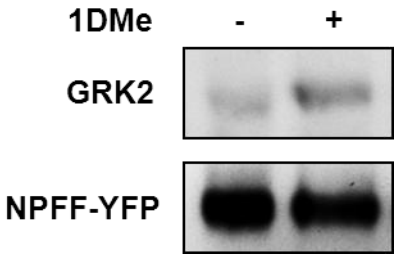
Suppl. Fig. 2. 1DMe-induced recruitment of GRK2 by the $hNPFF_2$ receptor. An equivalent number of cells were treated for 2 min at room temperature with buffer alone or 1µM 1DMe, followed by reversible cross-linking, solubilization and immunoprecipitation of the $hNPFF_2$ receptor using its YFP tag. Samples were immunoblotted with anti-GRK2 antibody followed by anti-GFP antibody to control the amount of immunoprecipitated receptor. The figure shows a representative result from 3 independent experiments.

Suppl. Fig. 3. Suppression of DAMGO and 1DMe-induced inhibition of adenylyl cyclase by pertussis toxin (PTX) treatment (100 ng/ml, 18h) in (SH_2-D9)MOP-YFP cells. The inhibitory effect of 1 µM DAMGO or 1DMe was tested after stimulation of cAMP production by 5 µM forskolin. Results are expressed as a percentage of the total amount of cAMP produced in the absence of receptor agonists.

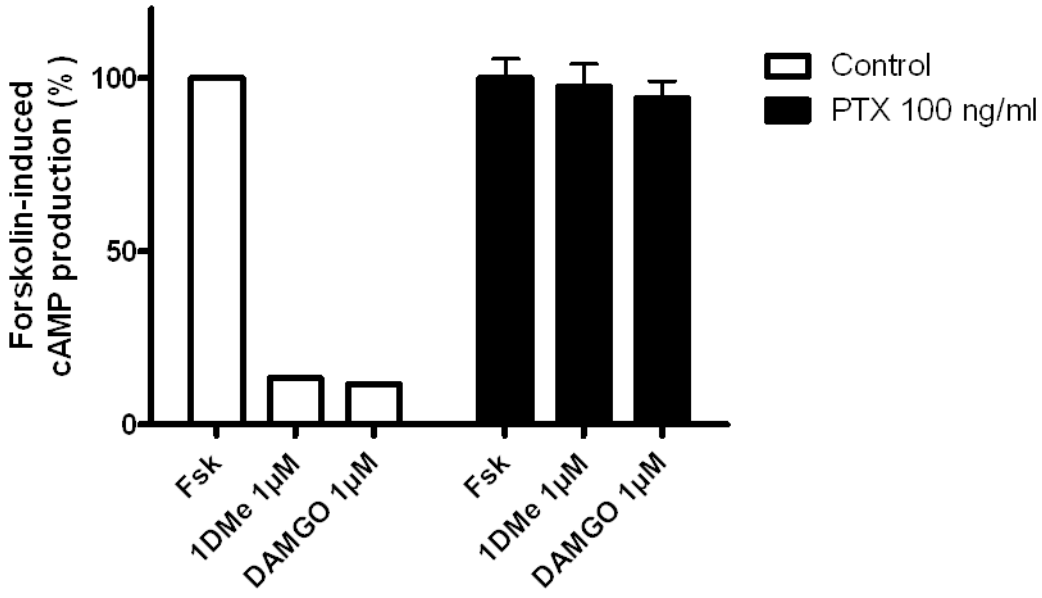
Suppl. Fig. 4. CID or ETD MS/MS spectra of phosphorylated peptides listed in Table 1.


A
total amount of MOP-YFP receptor (% of control)
200
150
100
50
0
C
DAMGO
DAMGO+1DMe
1DMe
Clonidine
B
1DMe (M)
0
10-8
10-7
10-6
10-5
P-MOP
actin
C
control
1DMe
DAMGO
P-MOP
actin

Suppl. Fig. 1



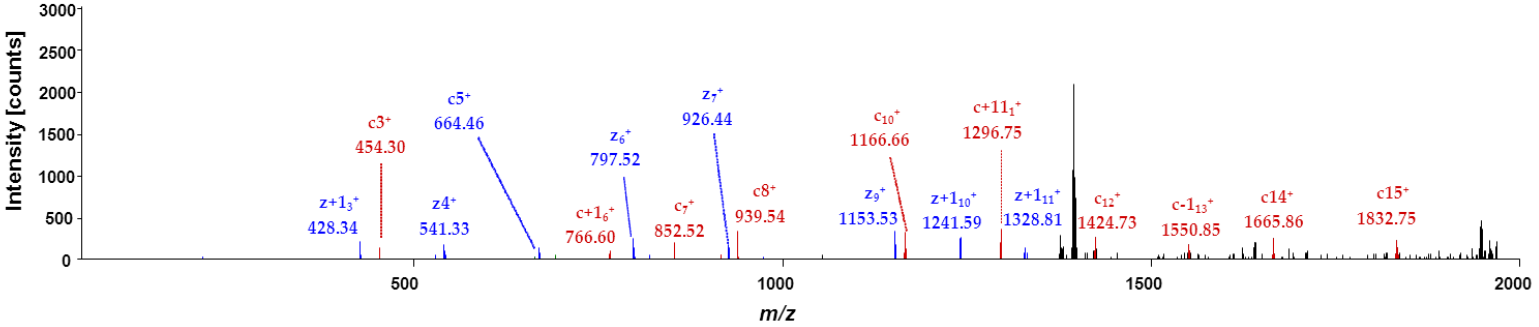
Suppl. Fig. 2


Forskolin-induced cAMP production (%)
100
50
0
Fsk
1DMe 1µM
DAMGO 1µM
Fsk
1DMe 1µM
DAMGO 1µM
Control
PTX 100 ng/ml

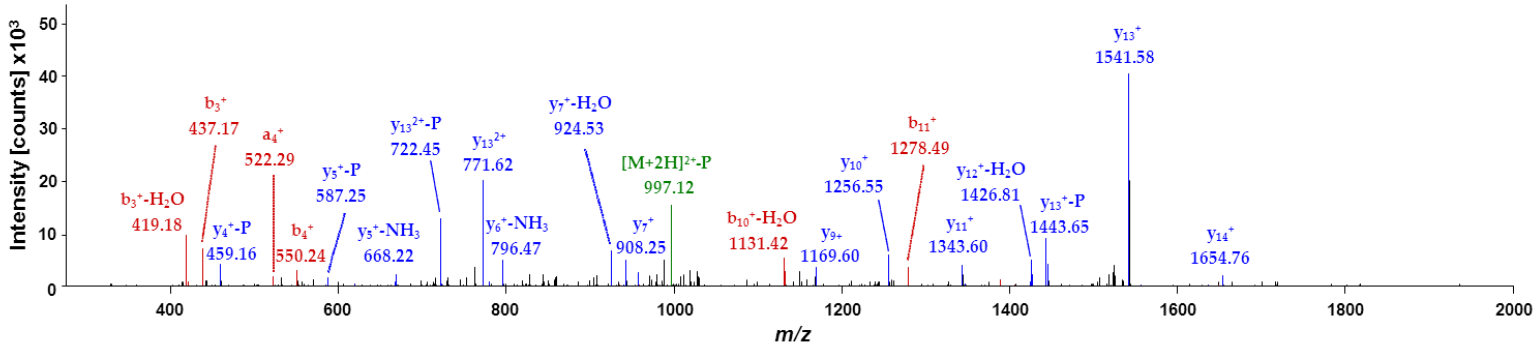
Suppl. Fig. 3

Suppl. Fig. 4

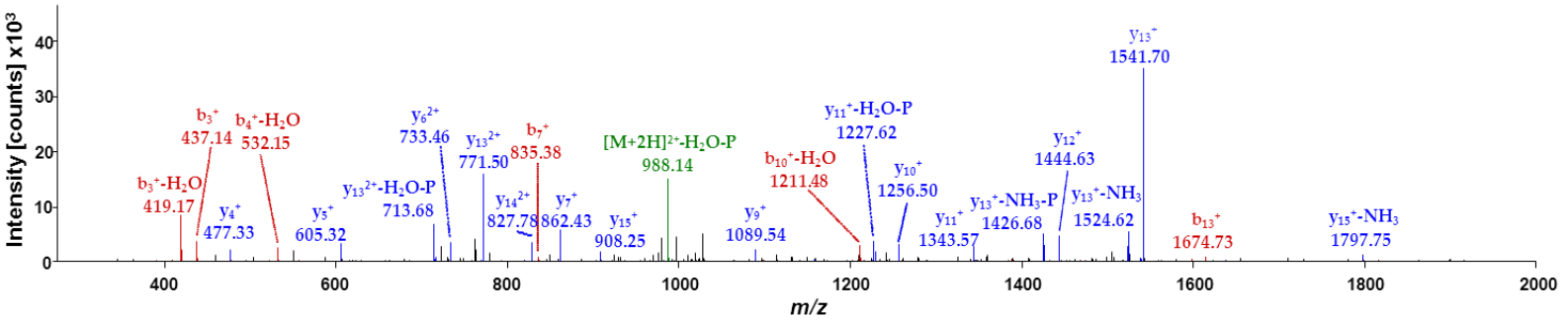
Sequence: 351- EFC$_{am}$IPTSSNIEQQNpSTR -367, Singly phosphorylated on S365
C$_{am}$: Carbamidomethyl on cysteine residue (57.0215 Da), pS: Phosphorylation on serine residue (79.9663 Da)
ETD spectrum, Charge: +3, Monoisotopic m/z: 697.6336 Da
Present in CTRL only



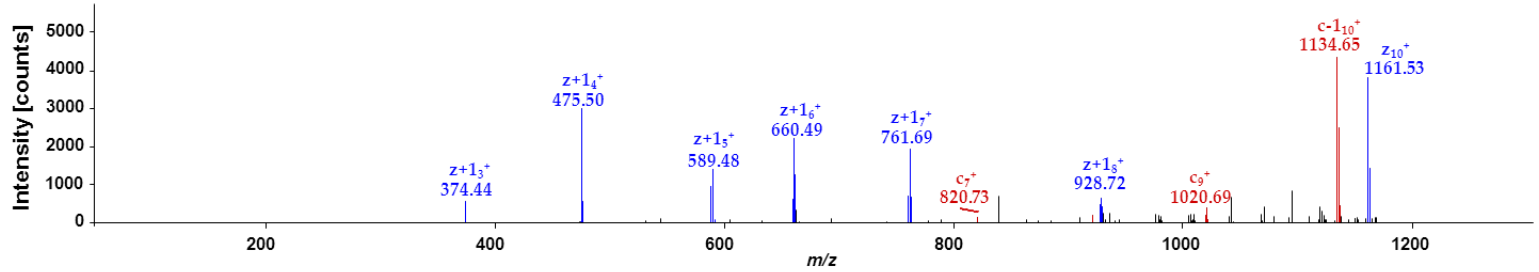
Sequence: 351- EFC$_{am}$IPTSSNIEQQNpSpTR -367, Singly Phosphorylated on S365 or T366
C$_{am}$: Carbamidomethyl on cysteine residue (57.0215 Da), pS or pT: Phosphorylation on serine or threonine residues (79.9663 Da), -P: loss of H_3PO_4 from sequence ions
CID spectrum, Charge: +2, Monoisotopic m/z: 1045.94653 Da
Present in DAMGO and 1DMe



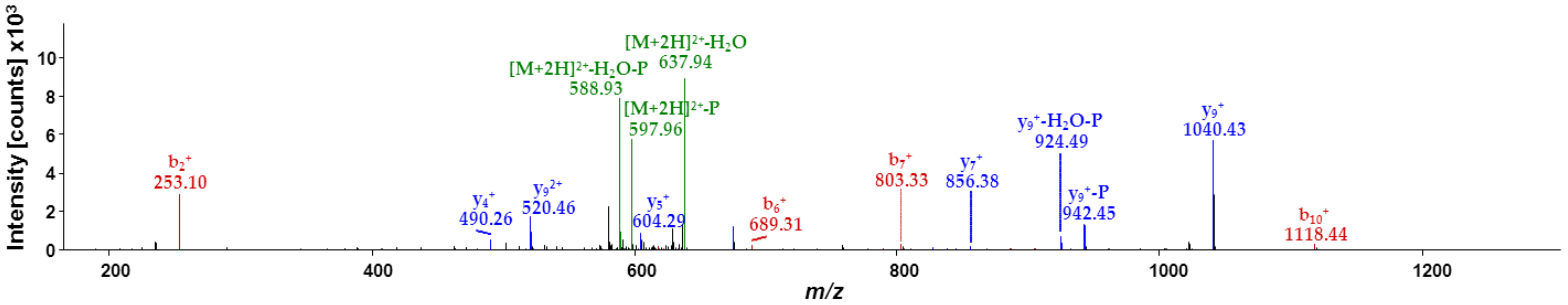
Sequence: 351- EFC$_{am}$IPTSpSNIEQQNSTR -367, Singly phosphorylated on S358
C$_{am}$: Carbamidomethyl on cysteine residue (57.0215 Da), pS : Phosphorylation on serine residue (79.9663 Da), -P: loss of H_3PO_4 from sequence ions
CID spectrum, Charge: +2, Monoisotopic m/z: 1045.94629 Da
Present in DAMGO only



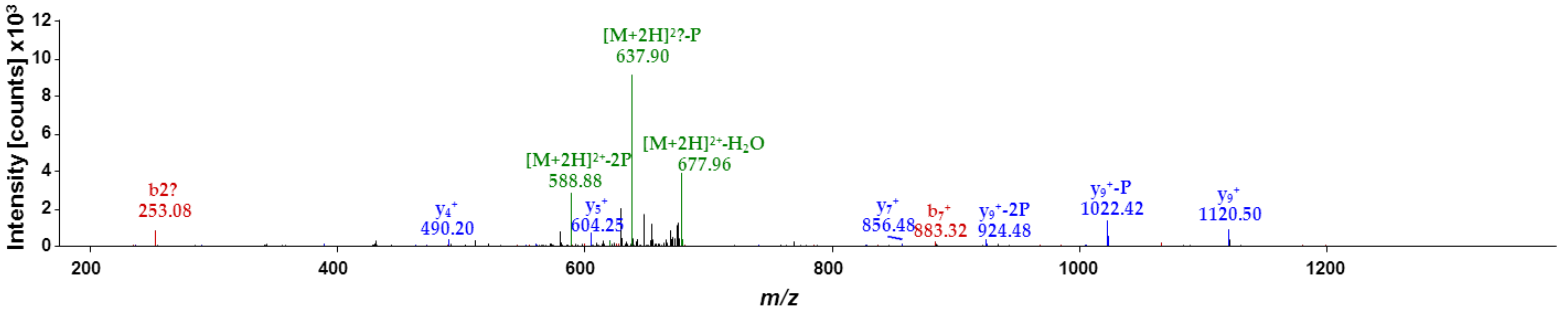
Sequence: 374 - DHPpSTANTVDR -384, Singly phosphorylated on S377
pS : Phosphorylation on serine residue (79.9663 Da)
ETD spectrum, Charge: +2, Monoisotopic m/z: 646.76715 Da
Present in CTRL, DAMGO and 1DMe



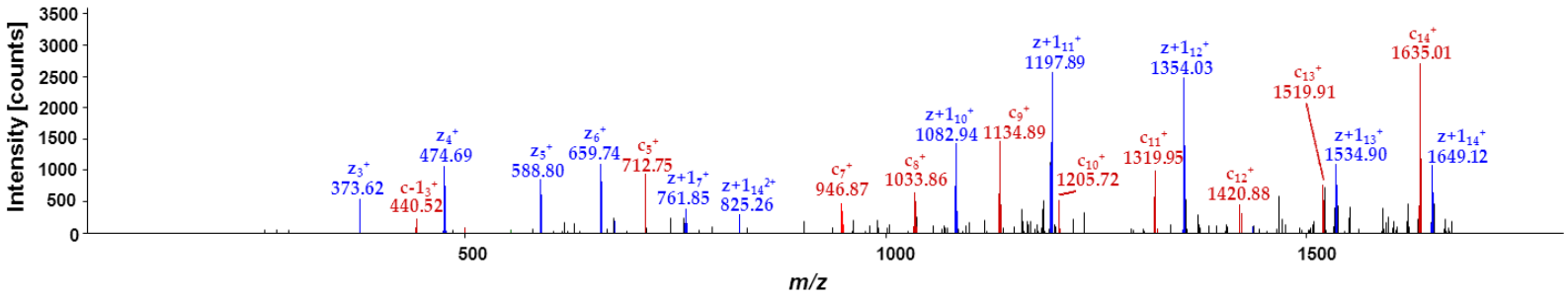
Sequence: 374 – DHPSpTANTVDR - 384, Singly phosphorylated on T378
pT : Phosphorylation on threonine residue (79.9663 Da), -P: loss of H_3PO_4 from sequence ions
CID spectrum, Charge: +2, Monoisotopic m/z: 646.76709
Present in CTRL, DAMGO and 1DMe



Sequence: 374 – DHPpSpTANTVDR - 384, Doubly phosphorylated on S377 and T378
pS and pT : Phosphorylation on serine and threonine residues (79.9663 Da), -P: loss of H_3PO_4 from sequence ions
CID spectrum, Charge: +2, Monoisotopic m/z: 686.75043 Da
Prensent in DAMGO and 1DMe



Sequence: 370 - QNpTRDHPSTANTVDR - 384, Singly phosphorylated on T372
pT : Phosphorylation on threonine residue (79.9663 Da)
ETD spectrum, Charge: +3, Monoisotopic m/z: 597.93073
Present in CTRL, DAMGO and 1DMe



Sequence: 370 - QNpTRDHPpSpTANTVDR - 384, Doubly phosphorylated on T372 and S377 or T378
pS and/or pT : Phosphorylation on serine and/or threonine residues (79.9663 Da)
ETD spectrum, Charge: +3, Monoisotopic m/z: 624.58606 Da
Present in CTRL, DAMGO and 1DMe