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Supplementary Materials for

Subcellular location defines GPCR signal transduction

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary Figures:

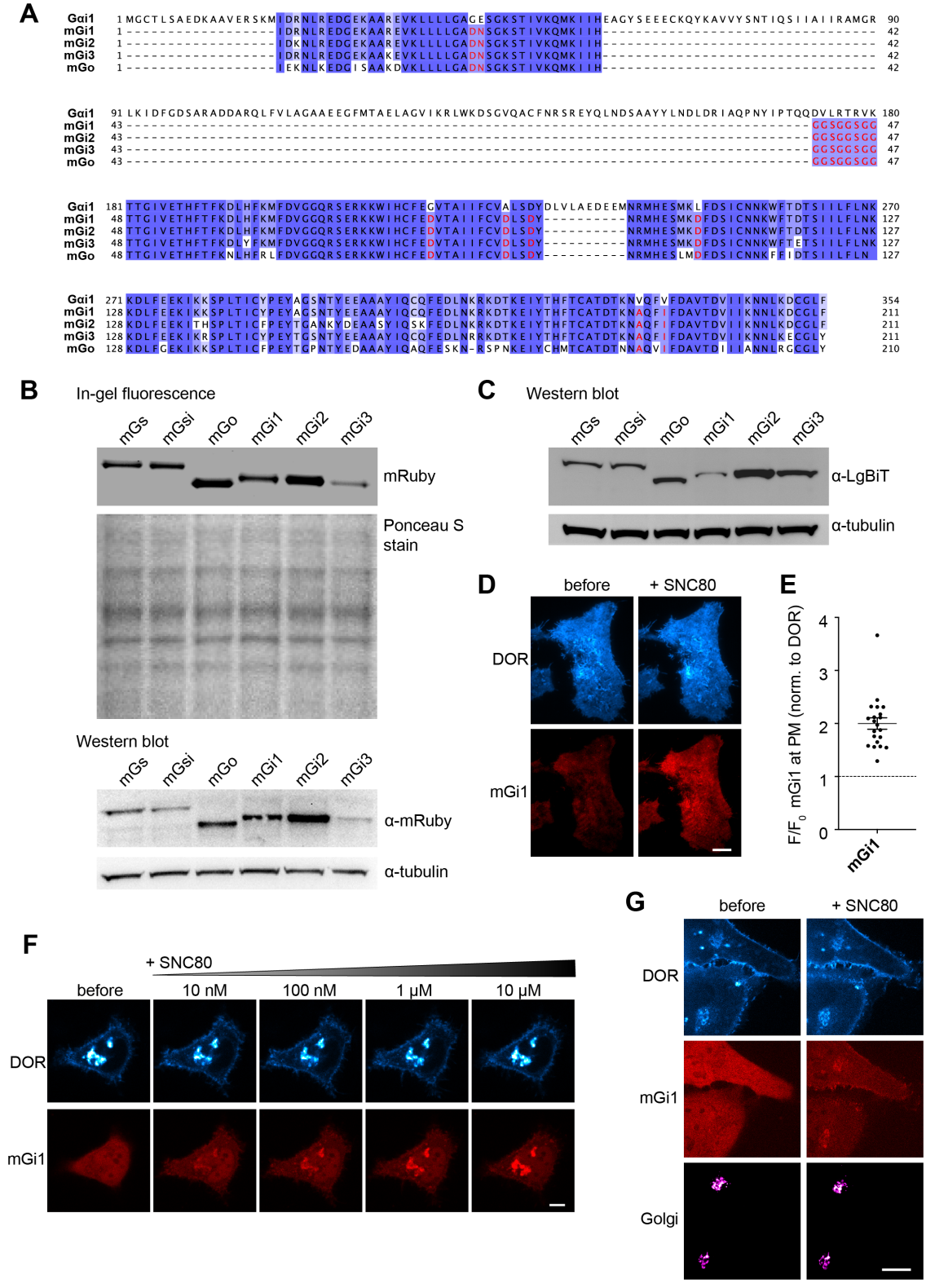


Figure S1: mG proteins derived from Gαi/o subunits are sensors of OR activation.
(A) Protein sequence alignment of full-length human Gαi with mGi1, mGi2, mGi3 and mGo. Red amino acids indicate stabilizing mutations in the mGi/o proteins. **(B)** mRuby2-mG (mGs, mGsi, mGo, mGi1, mGi2, mGi3) expression levels analyzed by in-gel fluorescence and western blot. Ponceau S stain of total proteins or tubulin was used as a loading control. **(C)** LgBiT-mG (mGs, mGsi, mGo, mGi1, mGi2, mGi3) expression levels assessed by western blot. Tubulin was used as a loading control. **(D)** TIR-FM images of HeLa cells expressing FLAG-DOR (cyan, surface-labeled with anti-FLAG M1-AF647) and

mGi1-mRuby2 (red), before and 5 min after adding 10 µM SNC80. Scale bar = 10 µm. **(E)** Quantification of mGi1 intensity in TIR-FM images acquired before and 5 min after SNC80 addition. N=3 with > 20 cells, mean ± SD. **(F)** Confocal images of HeLa cells, expressing DOR-SEP (cyan) and mGi1-mRuby2 (red) before and after adding increasing concentrations of SNC80 (10 nM, 100 nM, 1 µM, 10 µM). Scale bar = 10 µm. **(G)** Confocal images of transgenic HeLa cells stably expressing DOR-SEP (cyan) and transfected with mGi1-mRuby2 (red) and ManII-BFP (magenta) before and 5 min after adding 10 µM SNC80. Scale bar = 10 µm.

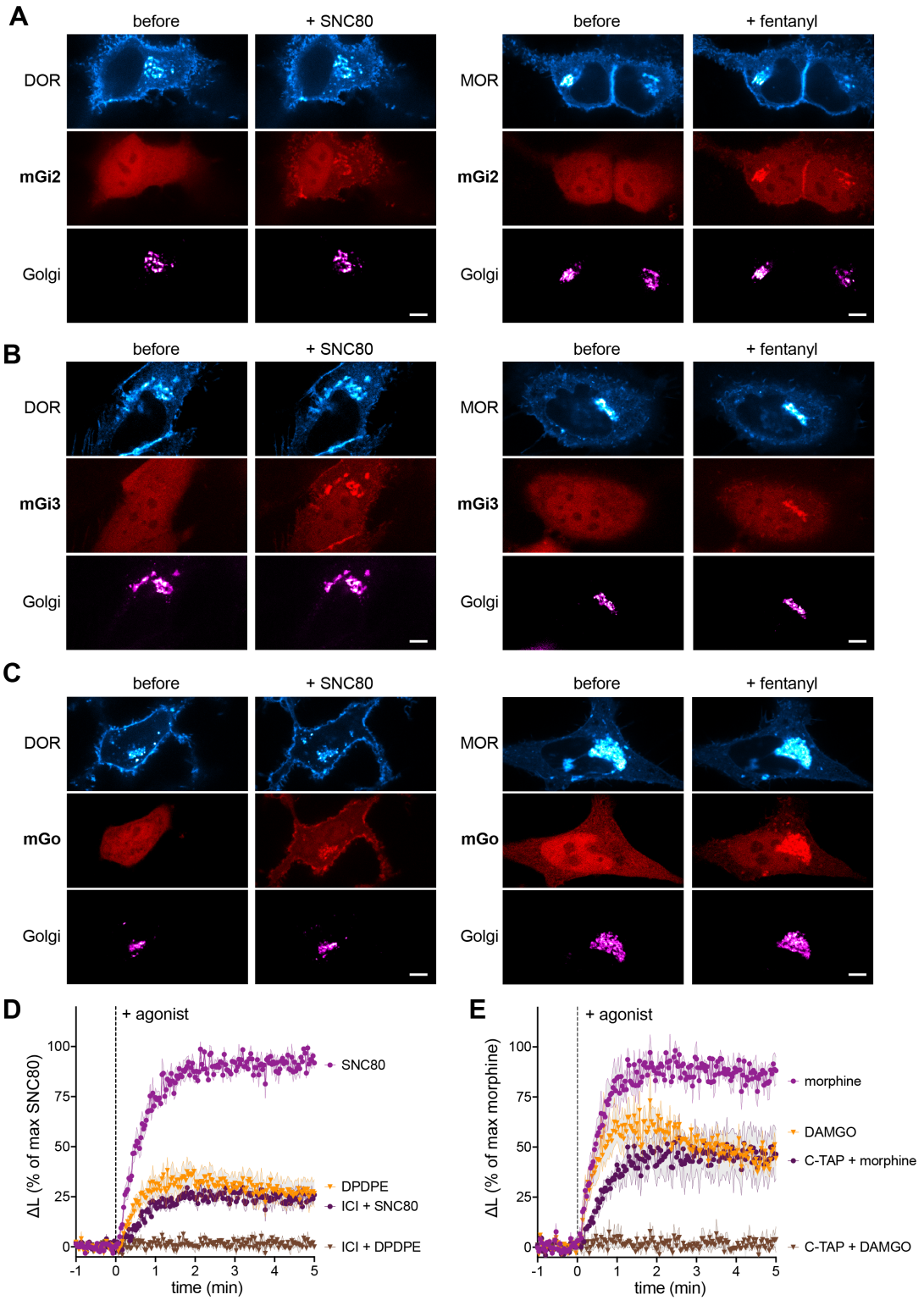


Figure S2: Activated Golgi-localized ORs recruit Gαi/o sensors.
(A), **(B)**, **(C)** Confocal images of HeLa cells, expressing DOR-SEP (left panels, cyan) or MOR-GFP (right panels, cyan), mRuby2-mG proteins (red), and ManII-BFP (magenta) before and 5 min after adding 10 µM SNC80 or 1 µM fentanyl. Scale bar = 10 µm. **(A)** mGi2-mRuby2, **(B)** mGi3-mRuby2, **(C)** mGo-mRuby2 expression. **(D)** Change in luminescence signal through agonist-induced interaction of mGi1-LgBiT with DOR-SmBiT (left panel) or MOR-SmBiT (right panel). Data normalized to maximum signal upon SNC80 (left) or morphine (right) addition. Left: 100 nM DPDPE or SNC80 in the absence or presence of ICI (100 µM). Right: 100 nM DAMGO or morphine in the absence or presence of CTAP (10 µM). N=3, mean ± SEM.

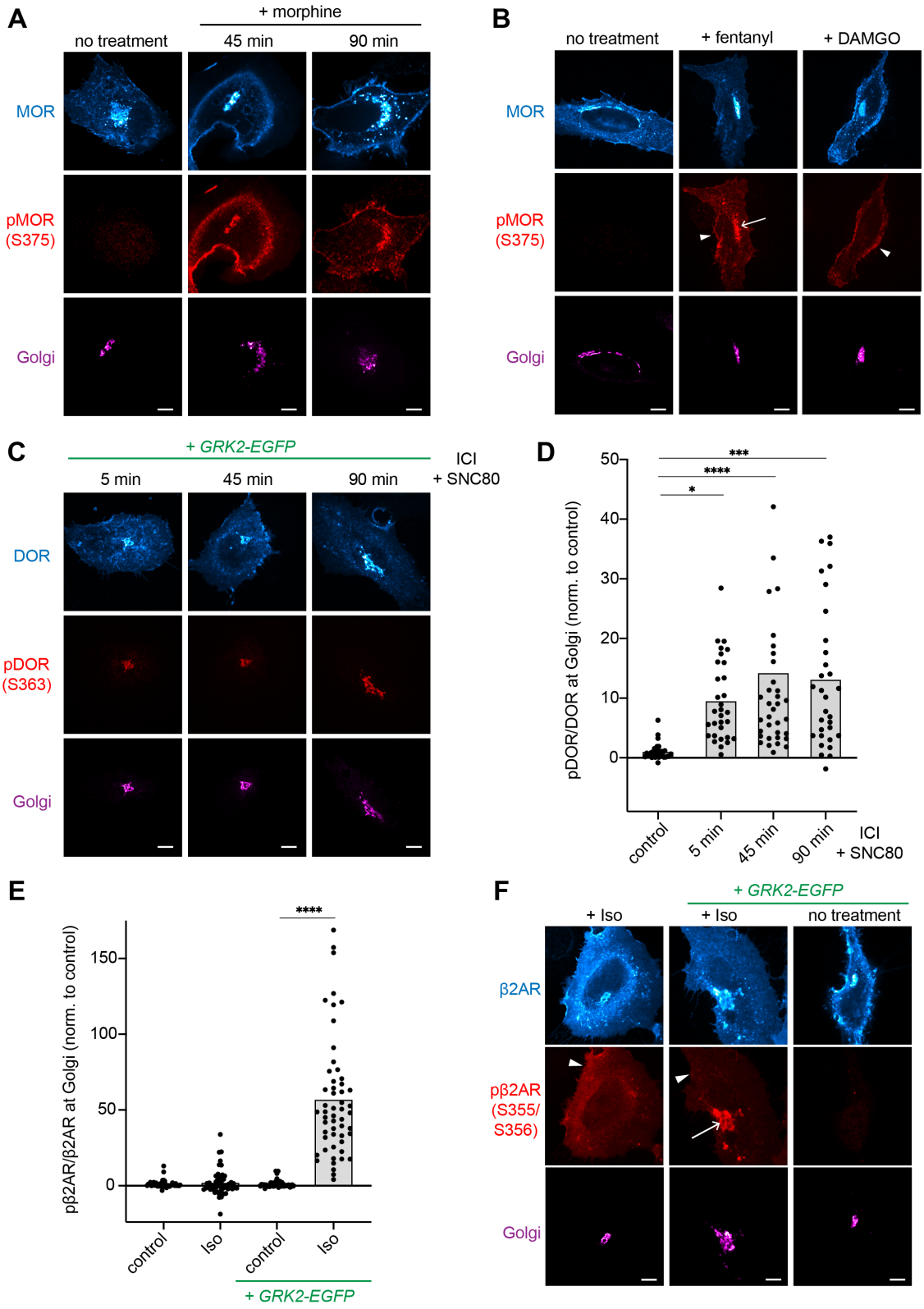


Figure S3: Activated GPCRs in the Golgi apparatus are regulated by phosphorylation.
(A) Confocal images of HeLa cells expressing FLAG-MOR and ManII-BFP (magenta). Cells fixed, permeabilized and immunolabeled with anti-FLAG (cyan) and anti-pMOR-S375 (red) antibodies. Cells untreated, or treated with morphine (10 μM) for 45 min or 90 min. Scale bar = 10 μm. **(B)** Staining and imaging of cells like in (A). Cells untreated, or treated with DAMGO (10 μM) or fentanyl (1 μM) for 5 min (quantification shown in Figure 2C). Scale bar = 10 μm. Arrow depicts pDOR at Golgi, arrowheads depict pDOR at PM. **(C)** Confocal images of HeLa cells expressing FLAG-DOR and ManII-BFP (magenta). Cells were fixed, permeabilized and immunolabeled with anti-FLAG (cyan) and anti-pDOR-

S363 (red) antibodies. Treatment with 100 μM ICI and 100 nM SNC80 for 5 min, 45 min, or 90 min. Cells were co-transfected with GRK2-GFP. Scale bar = 10 μm. **(D)** Quantification of pDOR/DOR fluorescence at Golgi (ManII-labeled) of cells transfected and stained as in (C). N=3 with >30 cells. *p<0.05, ***p<0.0005, ****p<0.0001 by ordinary one-way ANOVA. **(E)** Quantification of pβ2AR/β2AR fluorescence at Golgi (ManII-labelled). Cells transfected and stained as in (F). Cells treated with isoproterenol (Iso) for 5 min. Conditions with GRK2-EGFP co-expression are indicated. N=3 with >45 cells. ****p<0.0001 by ordinary one-way ANOVA. **(F)** Confocal images of HeLa cells expressing FLAG-β2AR and ManII-BFP (magenta). Cells immunolabeled with anti-FLAG (cyan) and anti-pβ2AR-S355/S356 (red) antibodies. Treatment with 10 μM Iso for 5 min of cells with or without GRK2-GFP expression. Arrow depicts pβ2AR in the Golgi area, arrowheads depict pβ2AR at the PM. Scale bar = 10 μm.

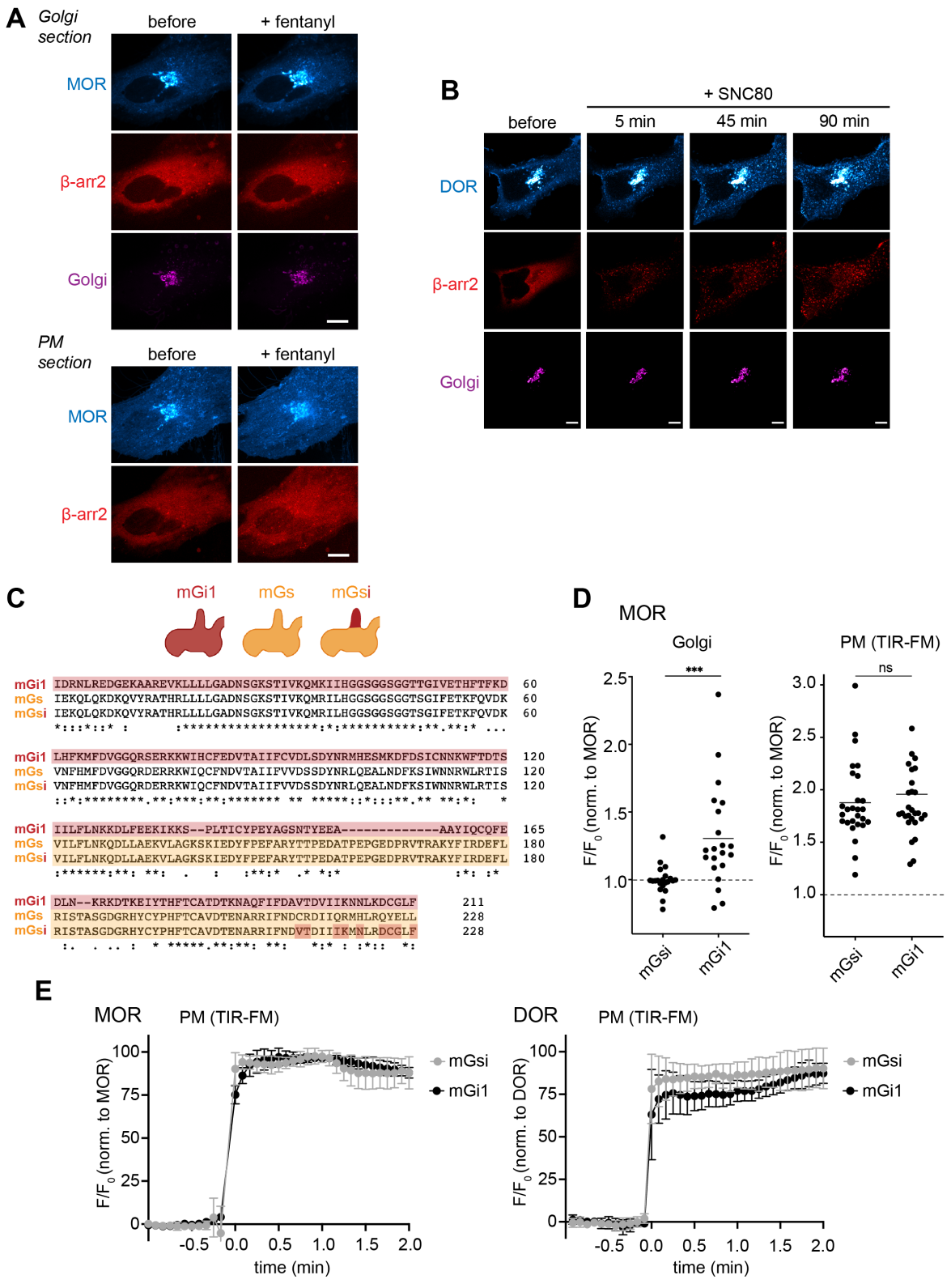


Figure S4: Location-selective OR binding to β-arrestin2 and mGsi.
(A) Confocal images of Hela cells, expressing GFP-MOR (cyan), β-arrestin2-mCherry (red), and ManII-BFP (magenta) before and 5 min after adding 1 µM fentanyl. 2 confocal sections of the same cell are shown. Top: focus on the Golgi, bottom: focus on PM. Scale bar = 10 µm. **(B)** Confocal images of HeLa cells, expressing SEP-DOR (cyan), β-arrestin2-mCherry (red), and ManII-BFP (magenta) before and 5 min, 45 min, and 90 min after adding 10 µM SNC80. Scale bar = 10 µm. **(C)** Top: Schematic showing differences and similarities between mGi1, mGs, and mGsi. Bottom: Protein sequence alignment of mGi1, mGsi, and mGs. **(D)** Quantification of mGsi-mRuby2 and mGi1-mRuby2 recruitment to MOR in

Golgi or PM. Left: Probe recruitment to MOR at Golgi as measured by confocal imaging. Right: Probe recruitment to the PM as measured with TIR-FM imaging. Same cells were imaged before and 5 min after 1 μM fentanyl addition. N=3 with > 15 cells, mean +/- SD. ***p<0.001 by unpaired t test. **(E)** Kinetic of mGsi (gray) and mGi1 (black) recruitment to ORs in the PM (left: MOR, right: DOR) measured with TIR-FM. Agonist added at t=0. 5 s between frames is shown. F_0=average fluorescence intensity prior to agonist. N=3, mean ± SEM.

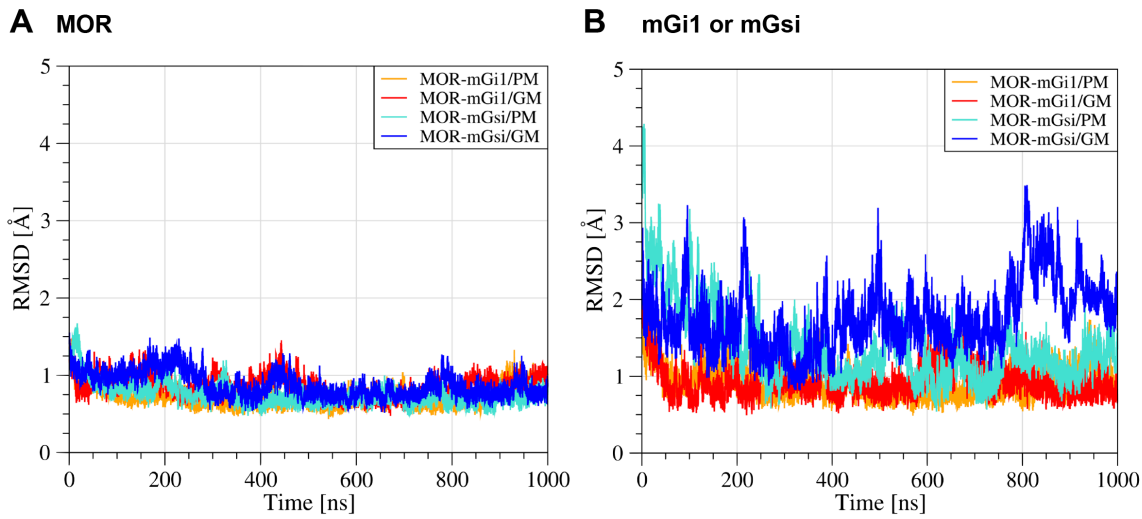


Figure S5: Dynamics and conformational stability of MOR, mGi1, and mGsi.
RMSD plots extrapolated from the MD simulations performed on the MOR–mGi1 and MOR–mGsi heterodimers in PM and Golgi membranes. **(A)** Time evolution of MOR's RMSD, measured on the secondary structure Cαs with respect to the average structure. **(B)** Time evolution of mGi1's and mGsi's RMSD, measured on their secondary structure Cαs with respect to each average structure.

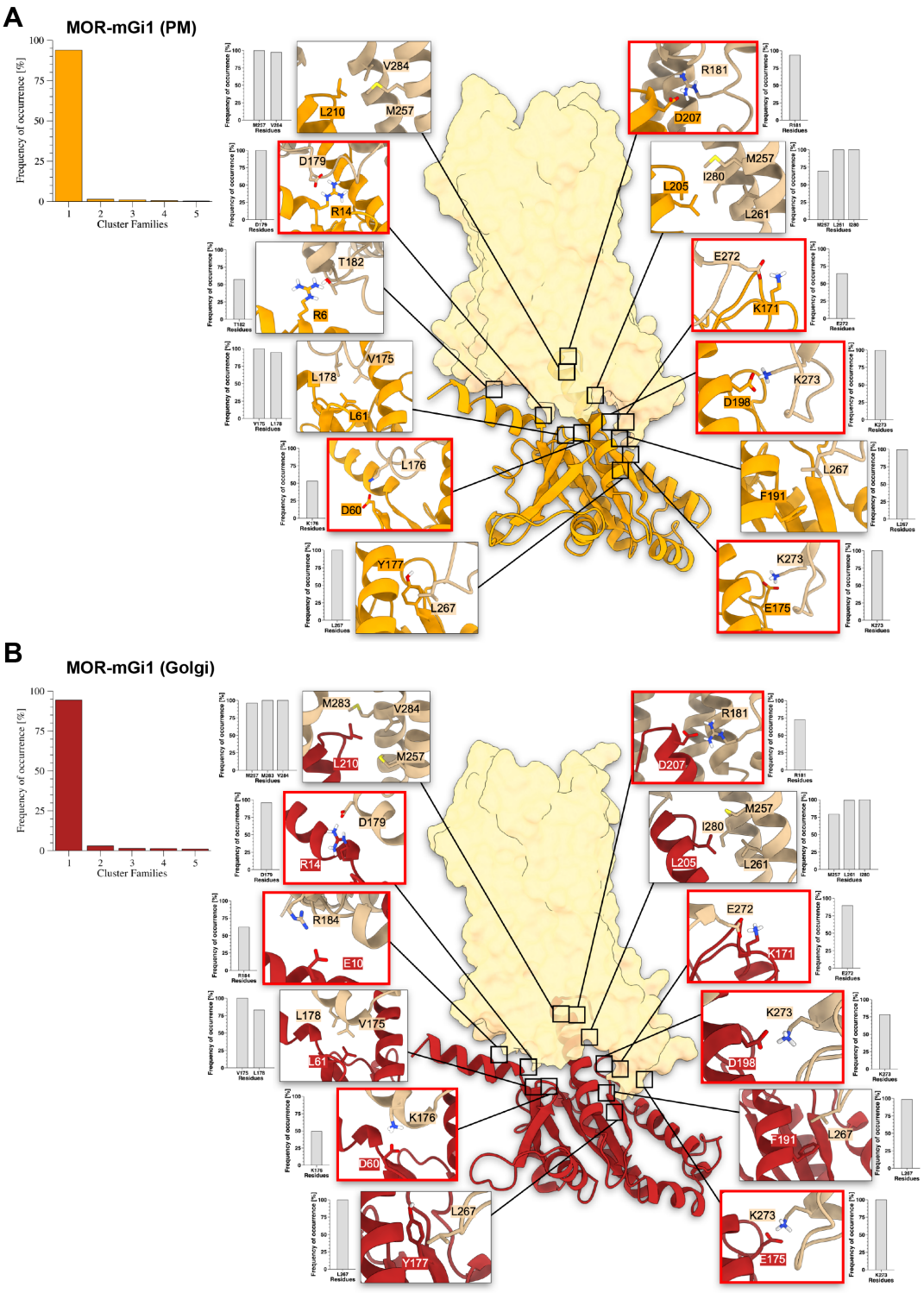


Figure S6: Structure analyses of PM-embedded and Golgi membrane-embedded MOR–mGi1 complexes. (A) The PM-embedded MOR–mGi1 heterodimer. Left: histogram of the conformational cluster analysis performed on the MD simulation. Right: MOR–mGi1 complex structure of the most populated cluster family. Insets show most relevant interactions between MOR and mGi1 and frequency of occurrence calculated from the MD simulation. Salt bridges highlighted with red box. **(B)** The Golgi membrane-embedded MOR–mGi1 heterodimer. Left: histogram of the conformational cluster analysis performed on the MD simulation. Right: MOR–mGi1 complex structure of the most populated cluster family. Insets show most relevant interactions between MOR and mGi1 and frequency of occurrence calculated from the MD simulation. Salt bridges highlighted with red box. MOR in transparent yellow and represented through solvent exposed surface. mGi1 in the PM or the Golgi lipid environment in orange and red, respectively.

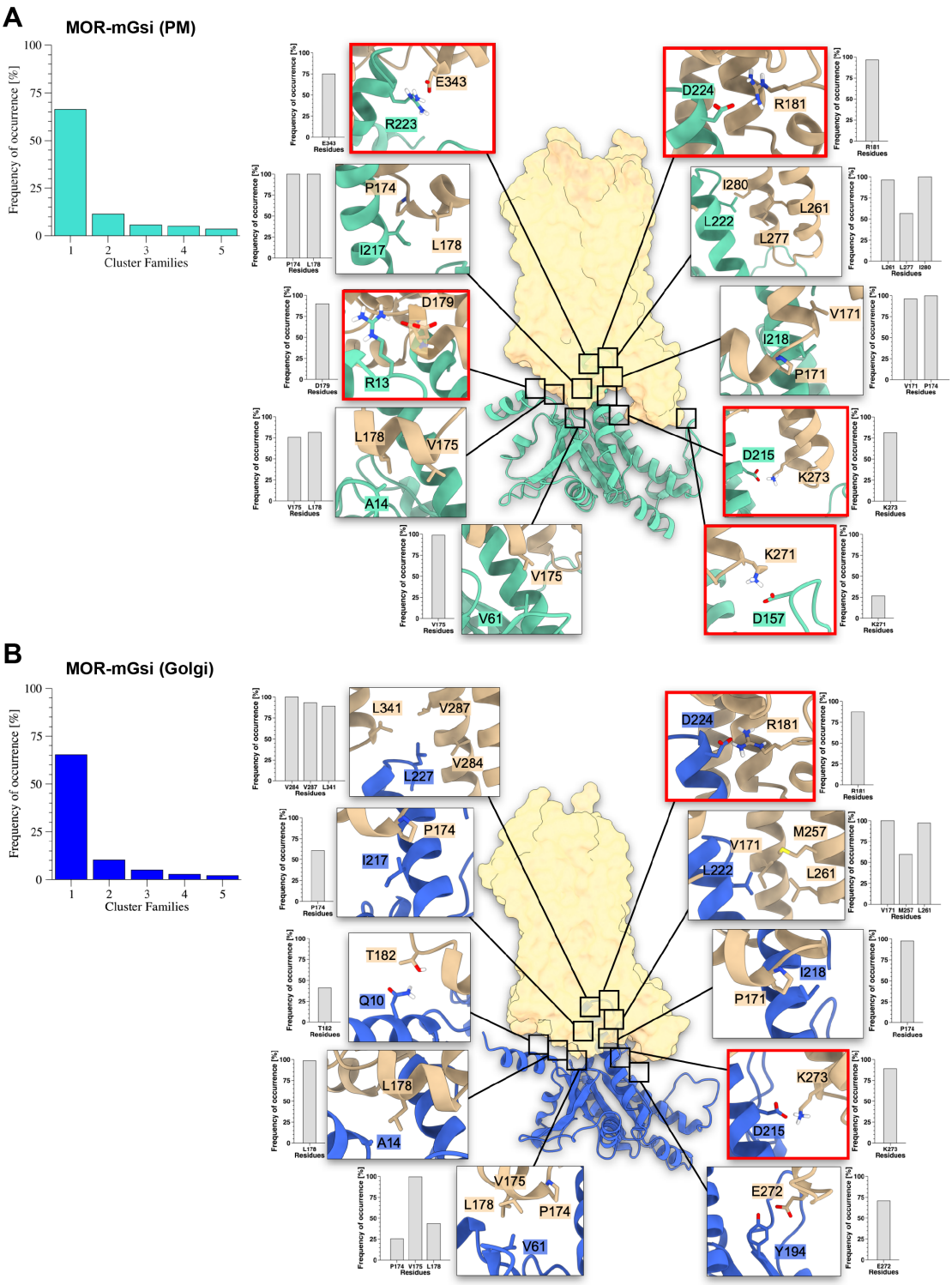


Figure S7: Structure analyses of PM-embedded and Golgi membrane-embedded MOR–mGsi complexes. (A) The PM-embedded MOR–mGsi heterodimer. Left: histogram of the conformational cluster analysis performed on the MD simulation; Right: MOR–mGsi complex structure of the most populated cluster family. Insets show most relevant interactions between MOR and mGsi and frequency of occurrence calculated from the MD simulation. Salt bridges highlighted with red box. **(B)** The Golgi membrane-embedded MOR–mGsi heterodimer. Left: histogram of the conformational cluster analysis performed on the MD simulation; Right: MOR–mGsi complex structure of the most populated cluster family. Insets show most relevant interactions between MOR and mGsi and frequency of occurrence calculated from the MD simulation. Salt bridges highlighted with red box. MOR in transparent yellow and represented through solvent exposed surface. mGsi in the PM or the Golgi lipid environment in teal and blue, respectively.

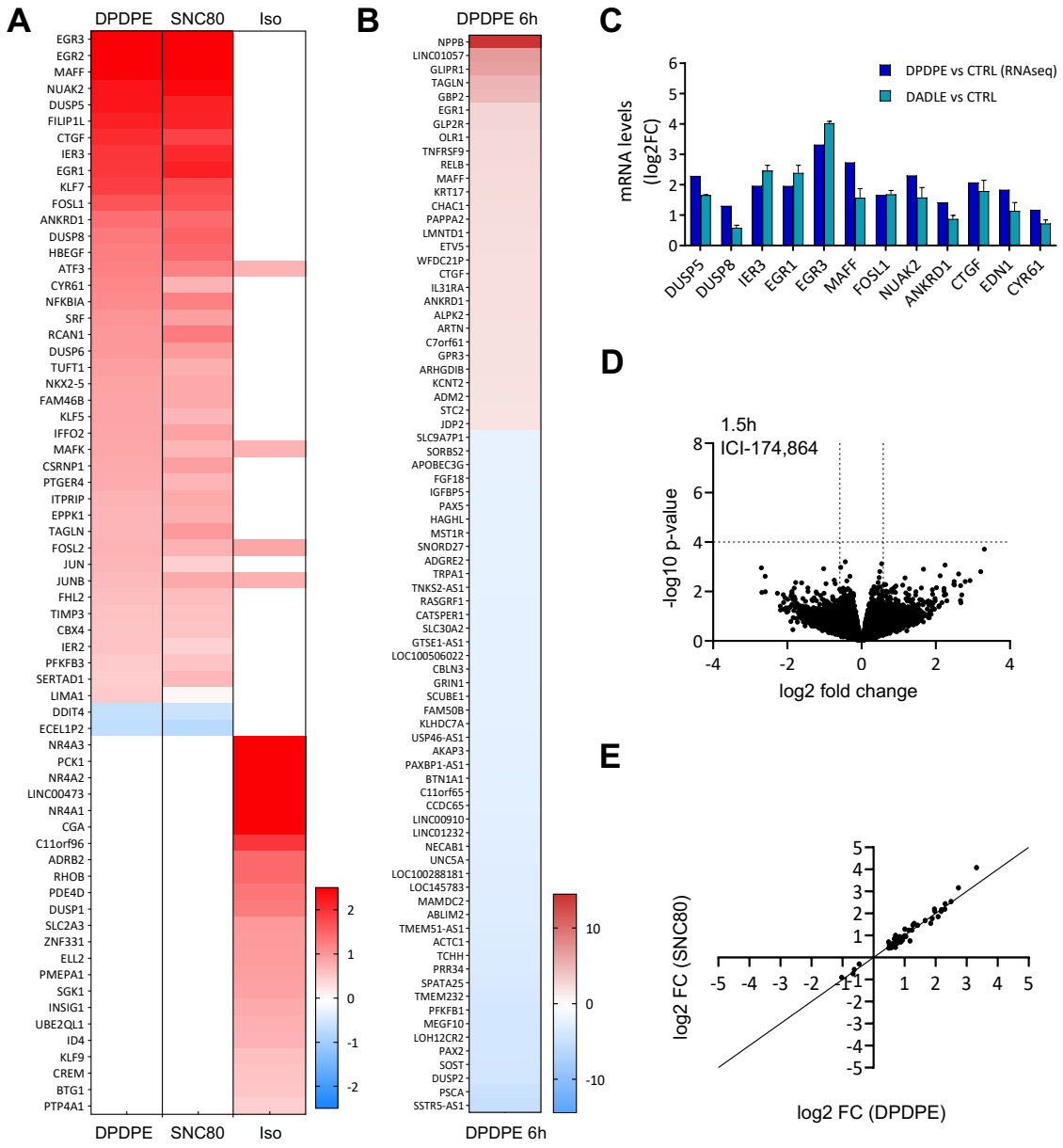
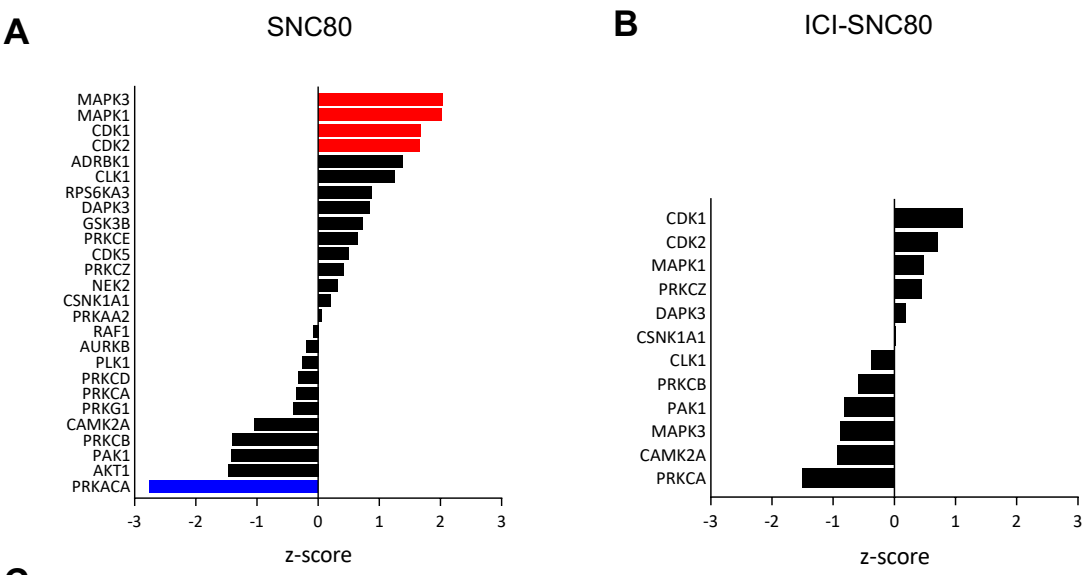


Figure S8: Characteristics of the DOR-driven transcriptional response.
(A) Heatmap of differentially expressed genes in HEK293-DOR cells treated with DPDPE (100 nM) or SNC80 (100 nM) for 1.5 h and upon β2AR activation with isoproterenol (Iso, 100 nM) for 1.5 h. Upregulated genes are shown in red (adj. p-value < 0.05) and downregulated genes are shown in blue (adj. p-value < 0.05). **(B)** Heatmap of differentially expressed genes in HEK293-DOR cells treated with DPDPE (100 nM) for 6 h. Upregulated genes are shown in red (FC > 2, adj. p-value < 0.05) and downregulated genes are shown in blue (FC < -2, adj. p-value < 0.05). **(C)** mRNA levels by RT-qPCR of selected genes regulated in HEK293-DOR cells upon treatment with DADLE (100 nM) for 1.5 h, compared to RNA-seq data. Relative gene expression was determined using the delta-delta Ct method. GAPDH used as reference gene. Results presented as log2 fold-change over mock-treated control cells. N=2. **(D)** Volcano plots of differentially expressed genes in HEK293-DOR cells treated with ICI (100 μM) for 1.5 h versus mock-treated control cells. Mean differential gene expression from 3 replicates. Results presented as log2 fold-change. No genes are significantly changed in their expression (criteria as in Figure 6). **(E)** Scatter plot comparing the expression levels (log2FC) of regulated genes in HEK293-DOR cells after 1.5 h treatment with DPDPE (100 nM) or SNC80 (100 nM). The black line depicts x=y.



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Kinase gene	mS	Enrichment	m	z-score	p-value	FDR
MAPK3	0.202294	4.883589	42	2.032741	0.021039	0.391578
MAPK1	0.279388	6.744719	19	2.022413	0.021567	0.391578
CDK1	0.210357	5.078235	26	1.679512	0.046526	0.391578
CDK2	0.227257	5.486223	21	1.660408	0.048416	0.391578
PRKACA	-0.30154	-7.27952	17	-2.75711	0.002916	0.367384

Figure S9: Kinase-substrate enrichment analyses for regulated phosphosites.
(A), (B) Bar plots showing kinase activity predictions in HEK293-DOR cells treated with SNC80 **(A)** or ICI-SNC80 **(B)** as in Fig. 6A-D using significantly regulated phosphopeptides at both time points for each treatment condition. Results were generated using the KSEA app (PhosphoSitePlus + NetworKIN, NetworKIN score cutoff = 1, p-value cutoff = 0.05, substrate count cutoff = 5) and are presented as kinase z-score. Kinases predicted to have increased activity compared to control are shown in red, kinases predicted to have decreased activity are shown in blue. **(C)** Table summarizing the output data (kinases with p-value < 0.05) from the KSEA kinase prediction analysis upon SNC80 treatment presented in (A) (Kinase gene = kinase gene name, mS = mean Log2FC of all kinase substrates, Enrichment = background-adjusted value of the kinase mS, m = total amount substrates detected from the experimental dataset for each kinase, z-score = normalized score for each kinase, FDR = adjusted p-value with Benjamini-Hochberg correction).

Supplementary Movies:

Movie S1: mGi1 recruitment to Golgi-localized DOR following SNC80 addition.
Confocal image series of a HeLa cell, expressing DOR-SEP (cyan), mRuby2-mGi1 (red), and ManII-BFP (magenta). Total movie duration is 5 min with image acquisition every 5 sec. 10 µM SNC80 added at t=0 sec.

Movie S2: mGi1 recruitment to Golgi-localized MOR following morphine addition. Confocal image series of a HeLa cell, expressing MOR-SEP (cyan), mRuby2-mGi1 (red), and ManII-BFP (magenta). Total movie duration is 5 min with image acquisition every 5 sec. 10 µM morphine added at t=0 sec.

Supplementary Tables:

Table S1: Transcriptomics data.
Genes significantly regulated in HEK293-DOR cells treated with DPDPE (100 nM) for 1.5 h (tab 1) or 6 h (tab 2), or with SNC80 (100 nM) for 1.5 h (tab 3).

Table S2: Phosphoproteomics data and analyses.
Peptides with significantly higher or lower phosphorylation upon treatment with SNC80 for 5 min (tab 1: 'SNC80 5min') or 25 min (tab 2: 'SNC80 25 min'). Peptides with significantly higher or lower phosphorylation upon treatment with ICI-SNC80 for 5 min (tab 3: 'ICI-SNC80 5min') or 25 min (tab 4: 'ICI-SNC80 25min'). Significantly regulated peptides that overlap between SNC80 5min and 25min, or ICI-SNC80 5min and 25min, or SNC80 and ICI-SNC80 (tab 5: 'Common phosphopep'). Data mining of links between identified hits and opioid receptor function (tab 6: 'Relation to OR'). Tabs 7-10: Identification of upstream kinases using the KSEA App in ICI-SNC80 condition (tab 8: 'KSEA ICI-SNC80 results') or SNC80 condition (tab 10: 'KSEA SNC80 results'). Unique and pooled phosphosites regulated by ICI-SNC80 (tab 7: 'KSEA ICI-SNC80 input') or by SNC80 (tab 9: 'KSEA ICI-SNC80 input') used as input for kinase prediction. Clustering of SNC80 and ICI-SNC80 hits based on function (tab 11: 'Functional groups'). GO enrichment for the pooled ICI-SNC80 hits (tab 12: 'ICI-SNC80 GO term analysis) or pooled SNC80 hits (tab 13: 'SNC80 GO term analysis').