

Supplementary Figures

Figure S1. Related to Figure 1.

(A) Sequences of nanobodies tested in this study. Alignment shows residues that vary between individual nanobodies. Red arrows: interactions between Nb39 and MOR (Huang et al., 2015). (B) Fluorescent protein-nanobody (Nb33) fusion constructs used in this study for expression in HEK293 and HeLa cells (EGFP-C1) and primary striatal neurons (pCAGGS). (C) Alignment of murine MOR and DOR sequences. Green arrows: interactions between MOR and Nb39 (Huang et al., 2015).

Figure S2. Related to Figure 1.

EGFP-OR-sensor fluorescence intensity time course measured by TIR-FM time-lapse acquisition of HEK293 cells expressing OR-sensor and FLAG-MOR (black, n=3), control plasmid (red, n=3), or FLAG-muscarinic acetylcholine receptor (M2, blue, n=3). Cells were stimulated with DAMGO (1 μ M), or carbachol (10 μ M) by bath application. F_0 = average fluorescence signal before agonist addition. Average \pm sem.

Figure S3. Related to Figure 2.

(A) TIR-FM images of a time series of a HEK293 cell, co-expressing clathrin-light-chain (CLC)-DsRed, FLAG-MOR (surface-labeled with M1-AF647) and EGFP-OR-sensor, 3 min after DAMGO (10 μ M) treatment. Scale bar 10 μ m. Arrows indicate the clathrin-coated pits analyzed in kymograph presented in (B). (B) 3.4 min kymograph of the TIRF image series shown in (A), starting 20s after adding DAMGO (10 μ M). Top arrows indicate individual CCP initiation and budding events. Right arrows show location of CCPs corresponding to the CCPs highlighted in (A).

Figure S4. Related to Figure 4.

Confocal images of striatal neurons (12-14 DIV) expressing FLAG-DOR (surface-labeled with M1-AF647) and pmApple- or EGFP-OR-sensor, before and 15 min after adding (A) DADLE (10 μ M), (B) DPDPE (10 μ M), or (C) Deltorphine II (10 μ M). Scale bars: 10 μ m.

Figure S5. Related to Figure 5.

(A) Confocal images of HeLa cells, expressing FLAG-DOR. Cells were fixed, permeabilized, and immunolabelled with anti-FLAG (red) and anti-Giantin (grey) antibodies. The internal OR pool co-localizes with Golgi marker (arrows). (B) Confocal images of a time series of HeLa cells, expressing EGFP-OR-sensor, GalT-DsRed (not depicted), and FLAG-DOR (not depicted). OR-sensor localization is shown before and 20 s after adding: left: etorphine, middle: ARM390. Right: etorphine (1 min), followed by naloxone (30 s). Examples of image series used to determine kinetics of Golgi activation shown in (C). (C) Quantification and kinetics of EGFP-OR-sensor intensity at Golgi apparatus upon agonist or antagonist addition in HeLa cells, expressing OR-sensor, GalT-DsRed, and DOR. GalT-marked Golgi apparatus was used as quantification mask. Time series with 2 s intervals for non-peptide ligands or 15 s intervals for peptide antagonists (TIPPpsi, ICI174). 1 μ M etorphine n=4, 10 μ M ARM390 n=4, 1 μ M etorphine followed by 10 μ M naltrindole (ndole) n=4, or by TIPPpsi n=5, or by ICI-174 n=6. Average \pm sem. (D) Confocal images of a HEK cell, expressing EGFP-OR-sensor, GalT-DsRed and FLAG-DOR (not shown) before and after addition of SNC80 (10 μ M). (E) Ligand concentration-dependent recruitment of OR-sensor to DOR in the Golgi apparatus (n=4, average \pm sem). GalT was used as quantification mask in confocal time-lapse series and OR-sensor signal normalized to cytosolic pool. Normalization of EGFP-intensity values (range [0-1]). Regression curves with Hill slope of 1. EC50: SNC80: 45 nM, etorphine: 110 nM. (F) Confocal images of a time series of a HEK cell, expressing EGFP-OR-sensor, GalT-DsRed, and FLAG-MOR (not shown). Cell was treated with

DAMGO (10 μ M) for 90 min. Left: no overlap between OR-sensor and GalT. Right: addition of morphine (1 μ M) for 30 s produces recruitment of OR-sensor to the Golgi marked by GalT. All scale bars: 10 μ m.

Figure S5. Related to Figure 6.

(A) Soma of striatal neuron (14 DIV), expressing FLAG-MOR (surface-labeled with M1-AF647), GalT-DsRed, and OR-sensor (pseudocolored low to high intensity). OR-sensor distribution is depicted before agonist and 20 s after adding etorphine (1 μ M). **(B)** Striatal neuron (12 DIV), expressing FLAG-MOR (surface-labeled with M1-AF647), and OR-sensor, treated with etorphine (1 μ M) for 15 min. Boxed areas are displayed below. Region 1: Co-localization of internalized MOR and OR-sensor in puncta along dendrites. Region 2: OR-sensor is recruited to Golgi apparatus, surface-labeled receptors are not enriched in this location. Scale bars: 10 μ m.

Supplementary Movies

Movie S1. Related to Figure 1.

TIR-FM time series of a HEK293 cell expressing EGFP-OR-sensor (and MOR, not shown). Media changes to 1 μ M DAMGO (t = 110 s) or 10 μ M naloxone (t = 410 s) by perfusion. Time between frames 2 s. Total movie length: 11:30 min.

Movie S2. Related to Figure 2.

Confocal time series of a HEK293 cell, expressing FLAG-MOR (red, surface-labeled with anti-FLAG M1-AF555) and EGFP-OR-sensor (green). 10 μ M DAMGO was added at t=0 s. Time between frames 2 s. Total movie length: 10:30 min.

Movie S3. Related to Figure 2.

Confocal time series of a HEK293 cell, expressing FLAG-DOR (red, surface-labeled with anti-FLAG M1-AF555) and EGFP-OR-sensor (green). 10 μ M DADLE was added at t=0 s. Time between frames 5 s. Total movie length: 8:20 min.

Movie S4. Related to Figure 4.

Confocal time series of a striatal neuron (12 DIV), expressing FLAG-MOR (red, surface-labeled with M1-AF555) and EGFP-OR-sensor (grey). 10 μ M DAMGO was added at t=0 s. Time between frames 5 s. Total movie length: 16:30 min.

Movie S5. Related to Figure 5.

Confocal time series of a HEK293 cell, expressing FLAG-MOR (red, surface-labeled with anti-FLAG M1-AF555) and EGFP-OR-sensor (green). 1 μ M Etorphine was added at t=0 s. Time between frames 2 s. Total movie length: 11 min.

Movie S6. Related to Figure 5.

Confocal time series of a HEK293 cell, expressing MOR(μ OR)-GFP (green) and mCherry-OR-sensor (red). 1 μ M morphine was added at t=0 s. Time between frames 2 s. Total movie length: 2:30 min.

Movie S7. Related to Figure 6.

Confocal time series of a striatal neuron (14 DIV), expressing EGFP-OR-sensor (green) and GalT-DsRed (and FLAG-MOR, not depicted). 1 μ M morphine was added at t=0 s. Time between frames 2 s. Total movie length: 3:40 min.

