SUPPLEMENTAL DATA

HETEROMERIZATION OF THE MU AND DELTA OPIOID RECEPTOR PRODUCES LIGAND-BIASED ANTAGONISM AND ALTERS MU RECEPTOR TRAFFICKING.

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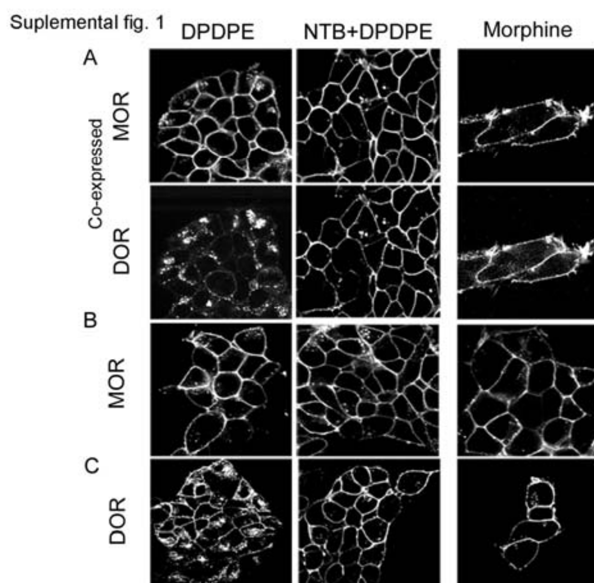
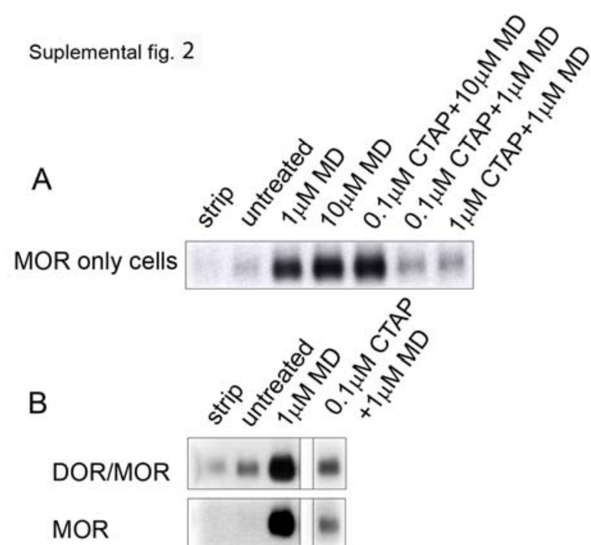
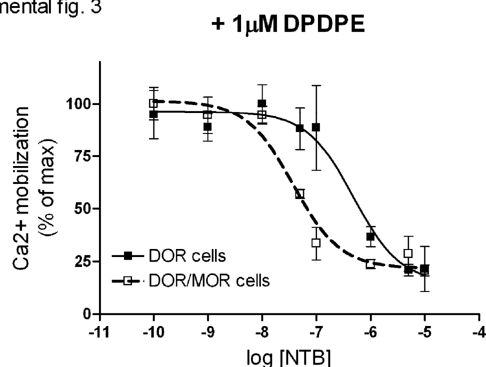


Supplemental Figure 1: *Effects of DPDPE and morphine on DOR/MOR endocytosis*. HEK293 cells stably expressing (A) FLAG-MOR and HA-DOR, (B) FLAG-MOR or (C) HA-DOR were incubated with antibody recognizing the N-terminal epitope tag(s) for 30 minutes to label surface receptors. Cells were then treated with 1 μM of the MOR agonist morphine (MS for morphine sulfate, left panels), for 30 minutes at 37 °C, 1 μM of the DOR agonist DPDPE for 30 minutes at 37 °C (middle panels) or 1 μM of NTB followed 20 minutes later with 1 μM of the DOR agonist DPDPE for 30 minutes at 37 °C. Cells were fixed and stained as in Methods. In cells co-expressing DOR and MOR, pictures were taken consecutively from dual color channels (green and red fluorescence). Images are representative examples of multiple independent experiments.



Supplemental Figure 2: *MOR antagonist CTAP inhibits methadone-mediated endocytosis of MOR homomers and DOR/MOR heteromers.* A) Endocytosis of MOR homomers from cells expressing only MOR was analyzed by biotin protection endocytosis assay as described in Methods. Cells were biotinylated then pre-treated with concentrations of CTAP as indicated for 20 minutes. Next, cells were treated with 1 μM or 10 μM of methadone (MD) for an additional 30 minutes. Endocytosed "protected" endocytosed receptors, were immunoprecipitated and resolved by SDS-PAGE gel. "Strip" lane demonstrates the efficiency with which biotin can be cleaved from surface receptors and represents the background. B) Endocytosis of DOR/MOR heteromers and MOR homomers from the same cell line was analyzed by biotin protection endocytosis assay as in (A). Cells were biotinylated, then pre-treated with 0.1 μM CTAP for 20 minutes. Next, cells were treated with 1 μM methadone (MD) for an additional 30 minutes. The fate of the protected endocytosed DOR/MOR heteromers (upper blot) and MOR homomers (lower blot) was assessed by serial immunoprecipitation followed by SDS-PAGE, as described in Methods. A representative immunoblot is shown.



Supplemental Figure 3: *NTB inhibits DPDPE-mediated signaling from DOR homomers and DOR/MOR heteromers.* Cells co-expressing DOR/MOR (open squares) or DOR only (closed squares) were pre-treated with increasing concentration of the DOR antagonist NTB for 20 minutes. Calcium release due to chimeric G protein $\Delta 6\text{-}G_{qi4}$-myr activation was measured in a Flex apparatus upon stimulation with the DOR agonist DPDPE (1 μM). Maximal effect for DPDPE (RFU): DOR (741 ± 95), DOR/MOR (734 ± 92). Shown are the mean ± S.E.M. n = 3 independent experiments.