SUPPORTING INFORMATION

Title: An immunocytochemical-derived correlate for evaluating the bridging of heteromeric mu-delta opioid protomers by bivalent ligands

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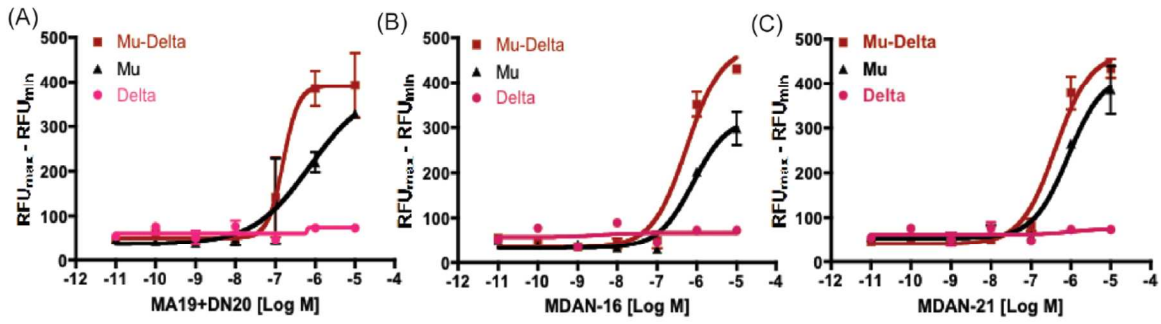
Figure S1



Supplemental Fig S1: Similar activation of mu and mu-delta opioid receptors by monovalent and bivalent ligands. Intracellular Ca^{2+} ion release was promoted by A) co-administered monovalent ligands,, MA-19 + DN-20: B) MDAN-16; and **(C** MDAN-21 were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric Giq-protein. Response for all the experiments was measured as Relative Fluorescence Units (RFU). The Y-axis plots the RFU values ± SEM (n = 8 - 12).

Supplemental Table 1. (a) Log EC_{50} and (b) ΔRFU values for Ca^{2+} release from HEK-293 cells stably expressing opioid receptors

(a) Log EC_{50}:

Ligand	Mu	Delta	Mu-Delta
MA-19+DN-20	- 6.15 ± 0.11	> - 6.00	- 6.80 ± 0.42
MDAN-16	- 6.10 ± 0.17	> - 6.00	- 6.25 ± 0.15
MDAN-21	- 6.07 ± 0.11	> - 6.00	- 6.39 ± 0.12

(b) ΔRFU:

Ligand	Mu	Delta	Mu-Delta
MA-19+DN-20	382.60 ± 67.70	73.99 ± 11.18	391.60 ± 39.19
MDAN-16	321.70 ± 26.88	67.37 ± 9.63	480.80 ± 40.80
MDAN-21	420.30 ± 25.80	76.71 ± 15.65	468.80 ± 23.33

Values presented as Mean ± SEM (n=8-12)

Supplemental methods

Immunocytochemistry: Two-color immunofluorescence was employed to analyze co-expression and internalization of mu- and delta-opioid receptors modified from a protocol previously described[1]. Briefly, HEK-293 cells coexpressing HA-delta and FL-mu were incubated with goat anti-HA (Abcam, Cat #ab9134) antibody and rabbit anti-FLAG (Abcam, Cat #1162) antibody at a final working concentration of 1:100 for 60 min at 4°C. After rinsing thrice with 50 mM PBS (pH 7.2), cells were incubated with or without ligands for 30 mins at 37°C to identify ligands that promote internalization. Primary antibodies were added to live unfixed cells so as to label receptors distributed on the plasma membrane only; fixing cells with formaldehyde kills cells makes their membranes permeable to antibodies and as a result both plasma membrane and cytoplasmic receptors will become labeled. Antibodies were added to live cells kept on ice to prevent constitutive internalization of receptors.

In antagonism experiments the delta antagonist, NTI, was incubated with cells 10-min prior to the addition of agonists. The cells were then rinsed again and were fixed with 4% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat #

SF40000-10; Neuromics, Inc.) containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

Cells. Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ, κ and mouse δ) were generated. HEK-293 cells co-expressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously [1]. Briefly, HEK-293 cells were cotransfected with HA-μ and FLAG-δ (μ-δ), HA-δ and FLAG-κ (κ-δ), HA-μ and FLAG-κ (μ-κ) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of μ-δ and κ-δ opioid receptor heteromers using co-immunoprecipitation . All the single and dual stable transfected cell lines were grown at 37°C and 10% CO_2 in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

Intracellular Ca^{2+} release assay. HEK-293 cells stably expressing opioid receptors obtained from Dr. Jennifer Whistler [2] were transiently transfected with a chimeric G_{α}-protein [3], Δ6-$G_{qi4\text{-}myr}$ at a concentration of 200 ng/20,000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). The DNA for the chimeric G-protein was the only DNA that was transiently transfected. The cell culture and calcium flux protocol was utilized as described previously [4]. Cells were grown to a confluency of approximately 2 million cells in a petridish. The cells were then

counted and DNA for the chimeric G-protein was added to a ratio of 200 ng/ 20,000 cells. Lipofectamine 2000 at a ratio of 1:2 wt/vol (DNA:Lipofectamine) was used for the transfection. The cells were then seeded 24 hours later into half area black 96 well plates (Corning) at 20,000 cells per well. The FLIPR calcium kit (Molecular devices) was used for the assay. Cells were incubated with a Ca^{2+} ion chelating dye from the kit, 48 hours after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as Relative Fluorescence Units (RFUs) and the time of the response was measured in seconds. A response window of 33 seconds after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. ΔRFU was computed for each concentration which was then plotted as a concentration response curve using non-linear regression. To incorporate well-well variability, four well replications were performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications where, each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, EC_{50} and RFU_{peak} values are all thus calculated from the four internal/dependent and three independent replications. Thus any variability due to transfection is contained in the error bars and has been accounted for.

References

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