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Supplemental information

Mu-opioid receptor selective superagonists produce prolonged respiratory depression

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Supplemental Information

Fig. S1 Assessment of MOR G protein coupling specificity, GRK2 effects, and BRET kinetics, related to Figure 1.

A) Baseline corrected net BRET curves for DAMGO-mediated G protein dissociation among non Gi/o/z subtypes are shown, with n=3 for each condition. B-D) The effect of GRK2 and a GRK inhibitor (compound 101) on DAMGO (B), fentanyl (C) and isotonitazene (D) β-arrestin2 recruitment by BRET. The following conditions are displayed: no co-transfected GRK (blue), no co-transfected GRK and addition of 100 μM compound 101 (purple), co-transfected GRK2 (red), and co-transfected GRK2 with the addition of compound 101 (orange). Drugs from each condition were normalized to their respective DAMGO control, and data are displayed as %DAMGO. Data represent mean and S.E.M performed in duplicate and n≥3 for all conditions. E) BRET G protein (GoA) dissociation assay curves are displayed for isotonitazene (red), and DAMGO (black) in the presence (dotted line) and absence (solid line) of co-transfected GRK2. Data represent mean and SEM performed in duplicate and n≥3 for all conditions. E-F) DAMGO mediated *β*-arrestin2 recruitment (F) and G protein (GoA) dissociation (G) after a 5 min (blue), 60 min (black), or 300 min (red) drug incubations are shown. G-H) Isotonitazene mediated *β*-arrestin2 recruitment (H) and G protein (GoA) dissociation (I) at the drug incubation time points listed above are shown. For E-H, drugs from each time-point were normalized to DAMGO from that respective time point, and data are displayed as %DAMGO for each time point.

Fig. S1 Assessment of MOR G protein coupling specificity, GRK2 effects, and BRET kinetics, related to Figure 1.

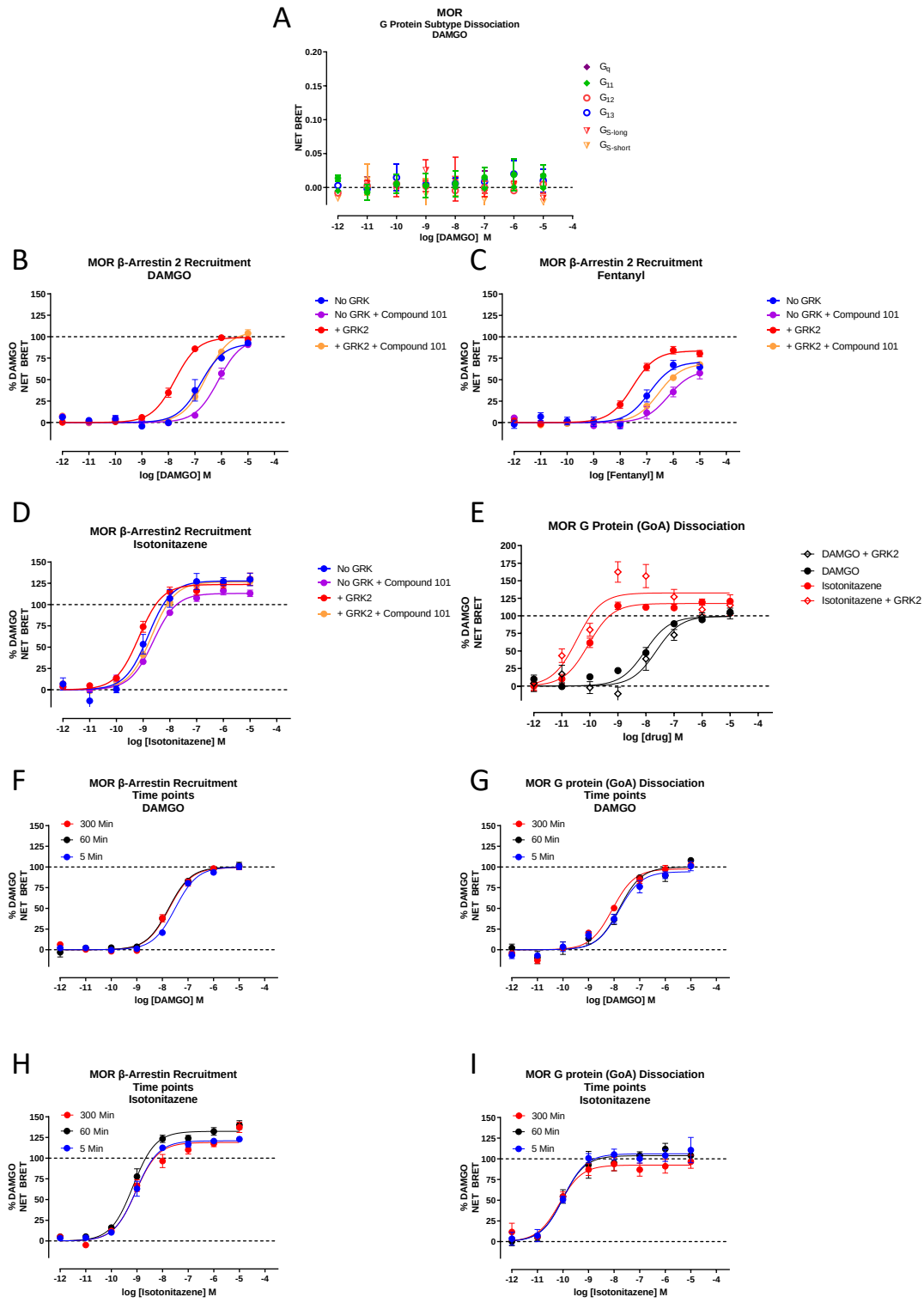

Fig. S2. Comparison of MOR mini-Gi, β-arrestin, and Gi/o/z protein subtype dissociation activities, related to Figures 1 and 2.

MOR G protein (GoA) dissociation (red), *β*-arrestin2 recruitment (blue), and mini-Gi protein recruitment (black) overlay graphs. DAMGO normalized dose-response curves are shown for DAMGO (A), isotonitazene (B), fentanyl (C) and *N*-desethyl isotonitazene. Data represent mean ± SEM for n≥3 for all compounds under each condition. Data is displayed as a percentage of the DAMGO net BRET with respect to each type of assay performed. E-H) DAMGO normalized net BRET curves for DAMGO (E) *N*-desethyl isotonitazene (F), metonitazene (G), and morphine (H) on MOR-mediated G protein dissociation among the Gi/o/z subtypes is shown (n≥3 for each condition).

Fig. S2. Comparison of MOR mini-Gi, β-arrestin, and Gi/o/z protein subtype dissociation activities, related to Figures 1 and 2.

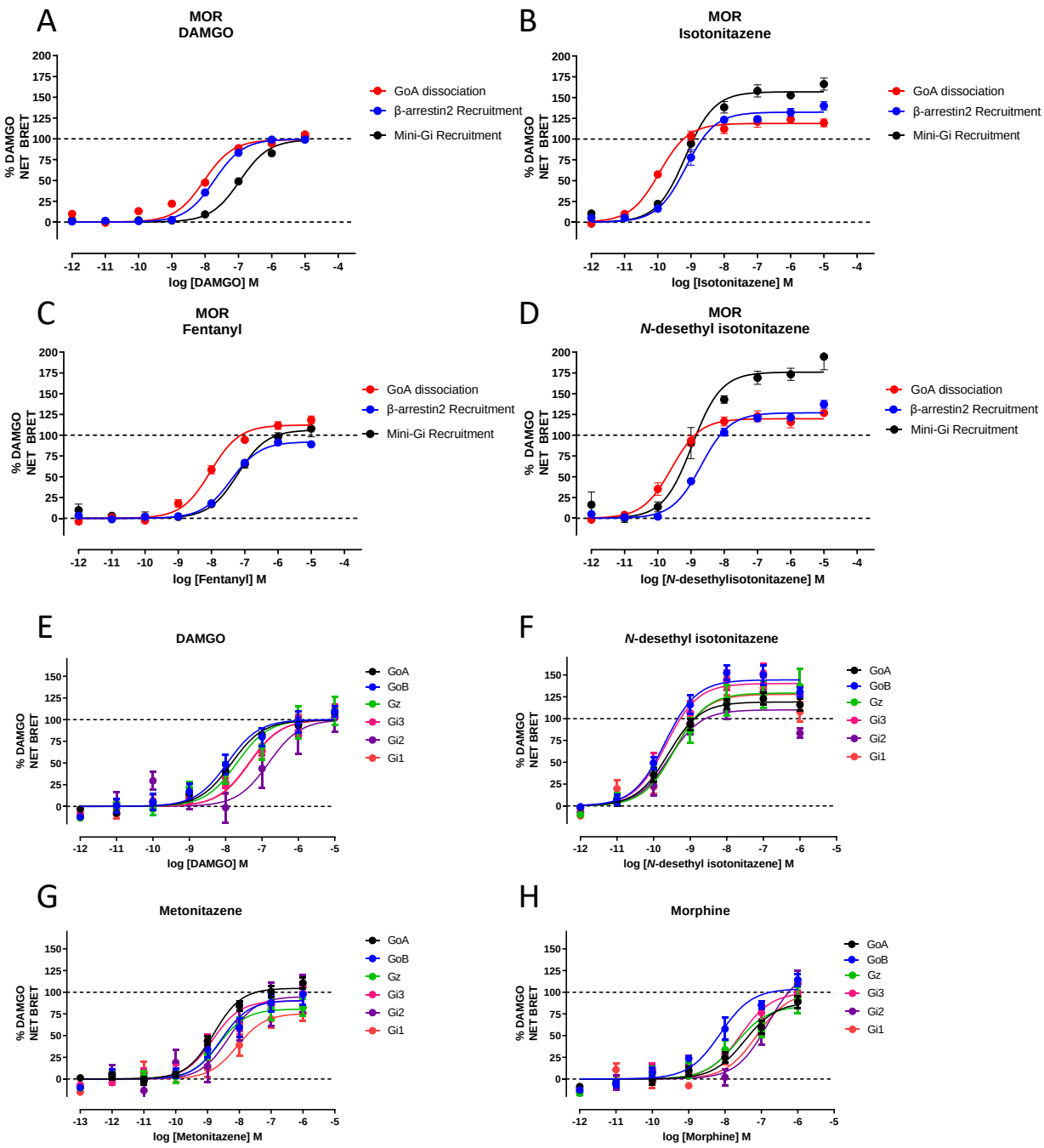

Fig. S3. MOR selectivity assessment for metonitazene and morphine, related to Figure 3. Dose-response curve overlays assessing selectivity at MOR, KOR, or DOR. Overlay graphs of dose response curves derived from the cAMP inhibition assay and G protein (GoA) BRET assay for metonitazene (A,B) and morphine (C,D) tested at MOR (red), KOR (black), and DOR (blue) receptors. The data were normalized to the control compounds DAMGO (MOR), DADLE (DOR), and salvinorin A (KOR), and are displayed as a percentage of control compounds maximum response. cAMP inhibition experiments assay data represent mean ± SEM for n≥3 experiments for each compound with each receptor. G protein (GoA) dissociation assay data represent mean and SEM performed in duplicate and n≥3 experiments for each compound with each receptor.

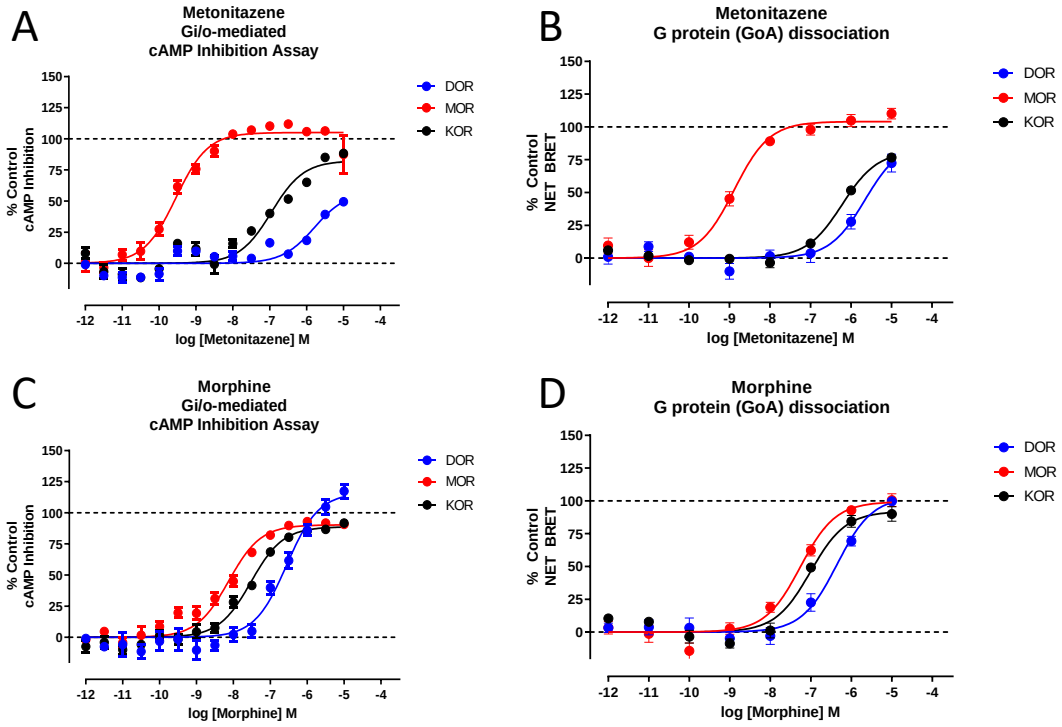

Fig. S4. Comparison of human to mouse MOR G protein dissociation activities, related to Figure 4.

Comparison of G protein dissociation activities between the human and mouse MOR. G protein (GoA) dissociation BRET dose-response curves at the human MOR (A) and mouse MOR (B) are shown, with efficacies shown as a percentage relative to DAMGO. The compounds DAMGO (black), fentanyl (blue), morphine (purple), isotonitazene (red), *N*-desethyl isotonitazene (brown), and metonitazene (orange) were tested at each receptor after a 60 min drug incubation time. All data represent mean ± SEM performed in duplicate and n≥3 experiments for all compounds at both receptors.

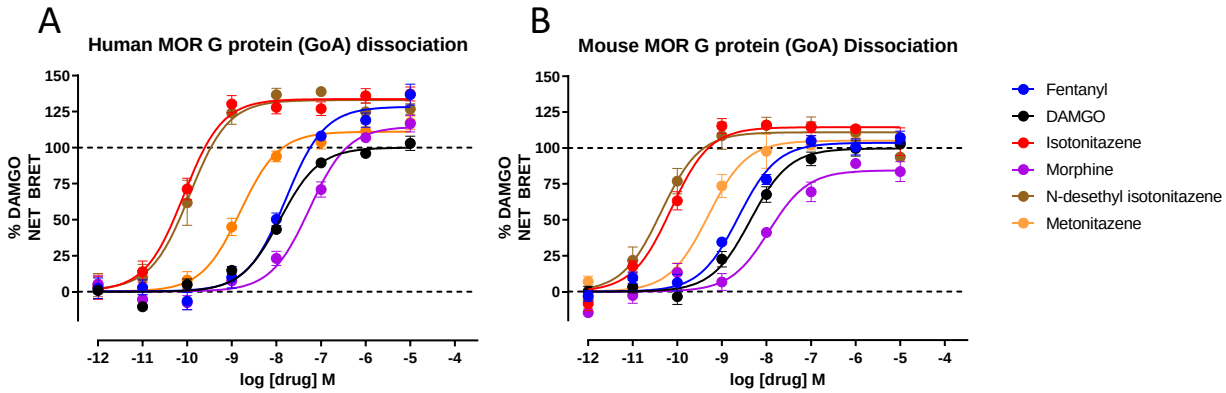

Table S1. BRET and GloSensor Assay Parameters, related to Figures 1-3.

Table of system, time, and temperature assay parameters for compounds tested in this study. N/A. not applicable.

Parameter	MOR G Protein Dissociation BRET	MOR β-arrestin2 Recruitment BRET	MOR GloSensor cAMP Inhibition	KOR GloSensor cAMP Inhibition	DOR GloSensor cAMP Inhibition
Transducer Pathway	G protein	β-Arrestin 2	Gi/o proteins	Gi/o proteins	Gi/o proteins
Cell Line	HEK 293T	HEK 293T	HEK 293T	HEK 293T	HEK 293T
Data Collection Time Point (min)	60	60	30	30	30
Temperature (°C)	37	37	25	25	25
Reference Ligand for Emax	DAMGO	DAMGO	DAMGO	Salvinorin A	DADLE
Reference Ligand for bias	DAMGO	DAMGO	N/A	N/A	N/A
Measured Process	Dissociation	Recruitment	cAMP	cAMP	cAMP
Measured Molecule 1	GαoA subunit (or others)	Mu Opioid Receptor	GloSensor luciferase	GloSensor luciferase	GloSensor luciferase
Measured Molecule 2	Gγ2 subunit (or others)	β-arrestin 2	cAMP	cAMP	cAMP
Co-expressed Molecule	β1 (or others)	GRK2	None	None	None
Signal Detection Technique	BRET	BRET	Luminescence	Luminescence	Luminescence