Supporting Information

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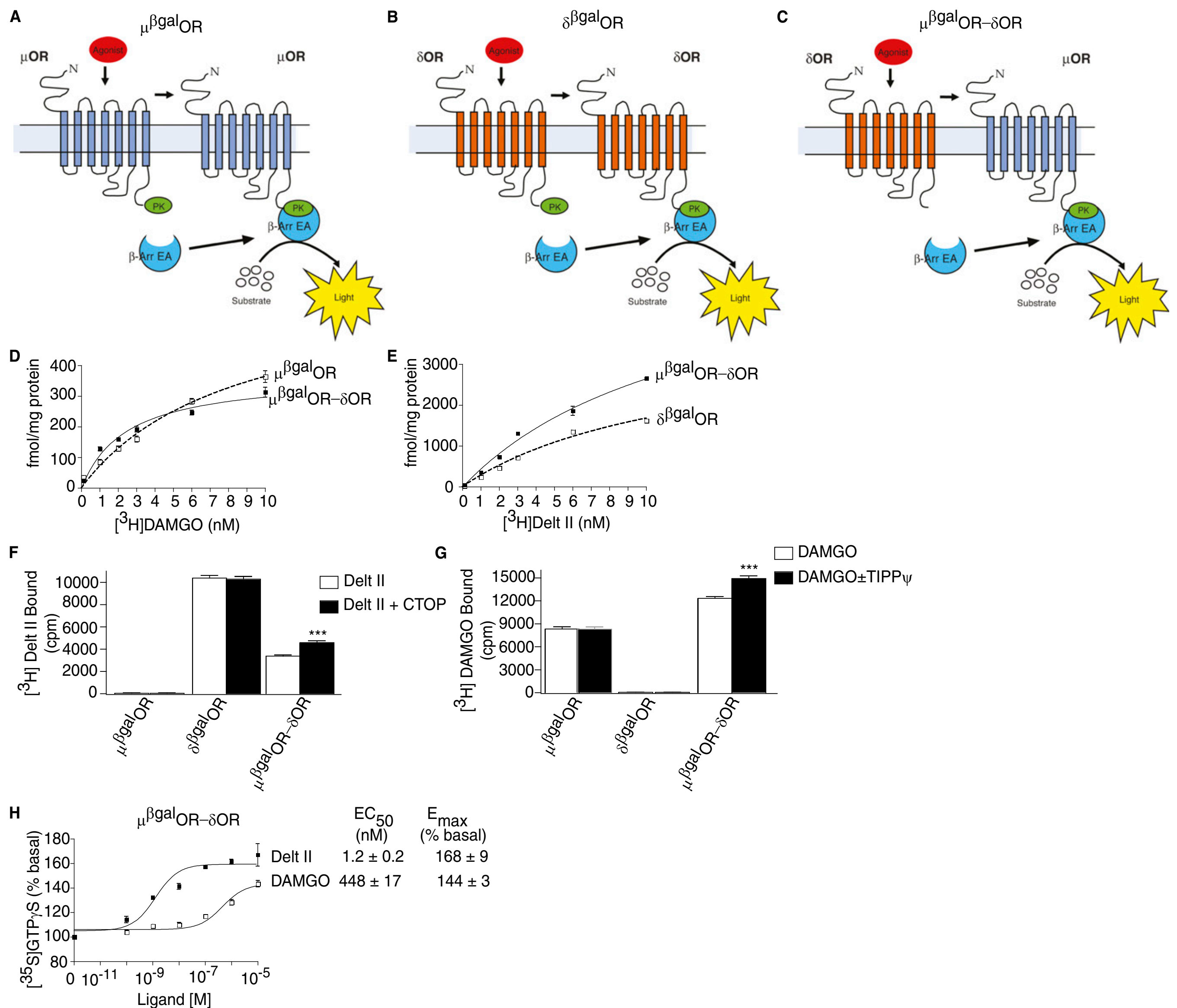

Fig. S1. Characterization of cells expressing $\mu^{\beta gal}$-opioid receptors ($\mu^{\beta gal}$OR), $\delta^{\beta gal}$-opioid receptors($\delta^{\beta gal}$OR), or $\mu^{\beta gal}$OR-δOR. (*A* and *B*) Schematic of homomer-mediated β-arrestin recruitment. Treatment of cells expressing either (*A*) μOR or (*B*) δOR tagged with ProLink/β-gal donor (PK; $\mu^{\beta gal}$OR or $\delta^{\beta gal}$OR, respectively) and β-arrestin tagged with a β-gal activator (EA) with receptor-selective agonists leads to recruitment of β-arrestin to the receptor and reconstitution of a functionally active β-gal, with activity that can be measured by the addition of an enzyme-specific substrate. (*C*) Schematic of heteromer-mediated β-arrestin recruitment. Treatment of cells expressing untagged δOR, μOR tagged with PK ($\mu^{\beta gal}$OR-δOR), and β-arrestin tagged with EA with a δOR-selective agonist leads to recruitment of β-arrestin to μOR and reconstitution of a functionally active β-gal, with activity that can be measured by addition of an enzyme-specific substrate. (*D* and *E*) Cells expressing $\mu^{\beta gal}$OR, $\delta^{\beta gal}$OR, or $\mu^{\beta gal}$OR-δOR (2×10^5 cells) were incubated with either (*D*) [^{3}H]DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) or (*E*) [^{3}H]deltorphin II ([^{3}H]Delt II; 0–10 nM final concentration) as described in *Materials and Methods*. Nonspecific binding was determined in the presence of 10 μM diprenorphine and was less than 10% of the total binding. (*F* and *G*) Cells expressing $\mu^{\beta gal}$OR, $\delta^{\beta gal}$OR, or $\mu^{\beta gal}$OR-δOR (2×10^5 cells) were incubated with (*F*) [^{3}H]Delt II (6 nM final concentration) in the absence or presence of the μOR antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP) (10 nM final concentration) or (*G*) [^{3}H]DAMGO (10 nM final concentration) in the absence or presence of the δOR antagonist H-Tyr-Tic[CH2NH]-Phe-Phe-OH (TIPPψ) (10 nM final concentration) as described in *Materials and Methods*. Nonspecific binding was determined in the presence of 10 μM diprenorphine and was less than 10% of the total binding. (*H*) Membranes (20 μg) from cells expressing $\mu^{\beta gal}$OR-δOR were subjected to a [^{35}S]GTPγS binding assay with either DAMGO or Delt II (0–10 μM final concentration) as described in *Materials and Methods*. Results represent mean ± SE ($n = 3$).

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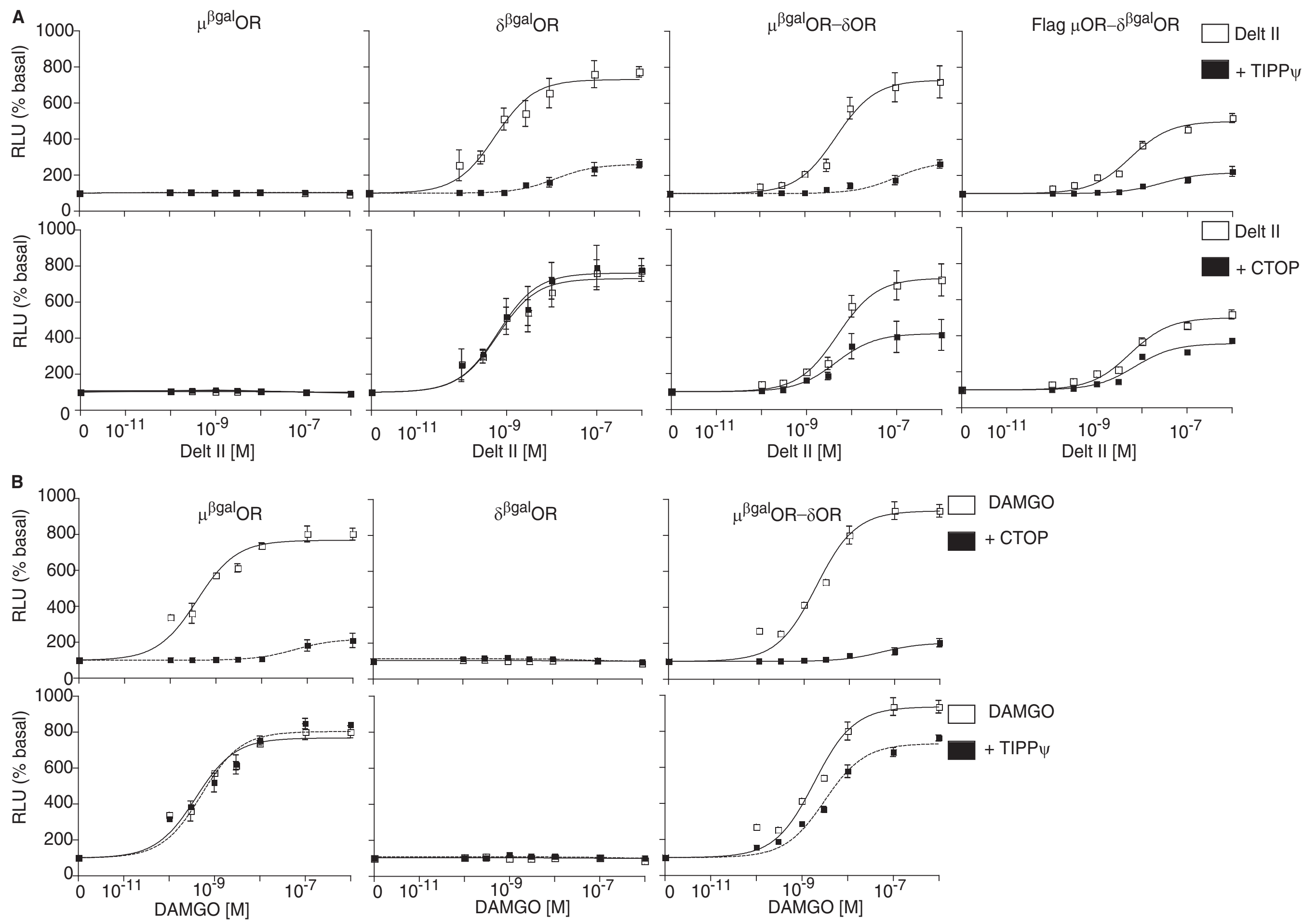

Fig. S2. Recruitment of β-arrestin by the δOR agonist Delt II or the μOR agonist DAMGO. (*A*) Cells expressing $\mu^{\beta gal}$OR, $\delta^{\beta gal}$OR, $\mu^{\beta gal}$OR-δOR, or Flag μOR-$\delta^{\beta gal}$ (20,000 cells/well) were plated into 96-well plates and subjected to a β-arrestin recruitment assay with the δOR agonist Delt II (0–1 μM final concentration) in the absence or presence of the δOR antagonist TIPPψ (10 μM final concentration) or the μOR antagonist CTOP (10 μM final concentration) as described in *Materials and Methods*. (*B*) Cells expressing $\mu^{\beta gal}$OR, $\delta^{\beta gal}$OR, or $\mu^{\beta gal}$OR-δOR (20,000 cells/well) were plated into 96-well plates and subjected to a β-arrestin recruitment assay with the μOR agonist DAMGO (0–1 μM final concentration) in the absence or presence of either the μOR antagonist CTOP or the δOR antagonist TIPPψ (10 μM final concentration) as described in *Materials and Methods*. Results are mean ± SE (n = 3–6). RLU, relative luminescence unit.

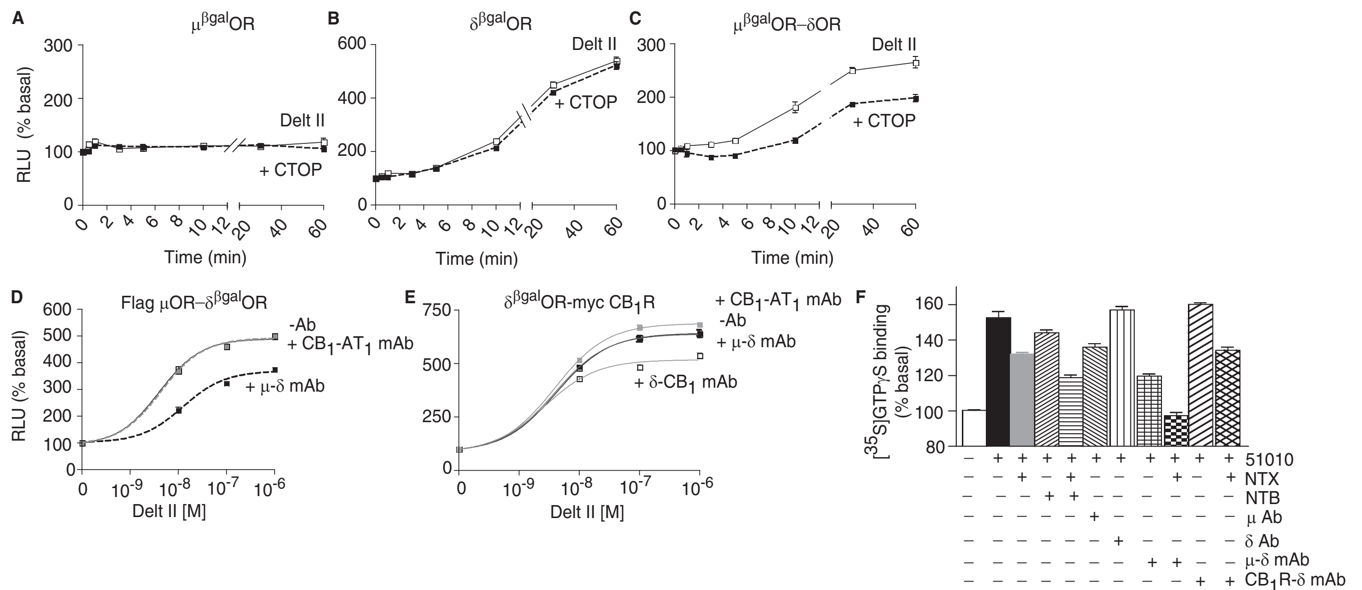

Fig. S3. Time course and effect of μOR-δOR heteromer-selective antibody on agonist-mediated β-arrestin recruitment. (*A*–*C*) Cells expressing (*A*) $\mu^{\beta gal}$OR, (*B*) $\delta^{\beta gal}$OR, or (*C*) $\mu^{\beta gal}$OR-δOR were treated with 1 μM Delt II in the absence or presence of 100 nM CTOP, and β-arrestin recruitment was measured as described in *Materials and Methods* at indicated time points. (*D*) Cells expressing Flag μOR-$\delta^{\beta gal}$OR (20,000/well) were treated with Delt II (0–1 μM) in the absence or presence of μ-δ or CB_1-AT_1 mAb (1 μg/well), and β-arrestin recruitment was measured as described in *Materials and Methods*. (*E*) Cells expressing $\delta^{\beta gal}$OR-myc CB_1R were treated with Delt II (0–1 μM) in the absence or presence of μ-δ, δ-CB_1, or CB_1-AT_1 mAb (1 μg/well), and β-arrestin recruitment was measured as described in *Materials and Methods*. (*F*) Spinal cord membranes (20 μg) from WT mice were subjected to a [^{35}S]GTPγS binding assay with CYM51010 (51010; 1 μM) in the absence or presence of μOR antagonist naltrexone (NTX; 10 μM), δOR antagonist naltriben (NTB; 10 μM), μ Ab (1 μg), δ Ab (1 μg), μ-δ mAb (1 μg) ± NTX (10 μM), or CB_1-δ mAb (1 μg) ± NTX (10 μM) as described in *Materials and Methods*. Results represent mean ± SE (n = 3–6).

Fig. S4. Effect of antagonists and μOR-δOR heteromer-selective antibody on antinociceptive activity of CYM51010. (*A*) Mice were administered s.c. with morphine (Mor) at the indicated doses, and antinociceptive activity was measured as described in *Materials and Methods*. (*B*) Mice were administered i.p. with vehicle, NTX, or NTB at the indicated doses 30 min before Mor administration, and antinociceptive activity was measured as described in *Materials and Methods*. (*C*) Mice were administered i.p. with vehicle, NTX, or NTB at the indicated doses 30 min before CYM51010 (51010) administration, and antinociceptive activity was measured as described in *Materials and Methods*. (*D*) Mice were intrathecally (i.t.) administered with Mor (0.3, 1, or 3 nmol), and antinociceptive activity was measured as described in *Materials and Methods*. *E* represents area under the curve (AUC) calculated from data in *D*. (*F–H*) Mice

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were administered i.t. with vehicle, control IgG (anti-Flag IgG; 1 μg), or μ-δ mAb (1 μg) 30 min before i.t. administration of (*F*) CYM51010 or (*G* and *H*) Mor, and antinociceptive activity was measured as described in *Materials and Methods*. *H* represents AUC calculated from data in *G*. (*I* and *J*) Mice were administered with either (*I*) Mor or (*J*) CYM51010 (10 mg/kg s.c.) one time daily for 8 d, and antinociceptive activity was measured daily as described in *Materials and Methods*. *K* represents peak antinociception calculated from data in *I* and *J*. (*L–O*) Mice were administered with either (*L*, *N*, and *O*) Mor or (*M–O*) CYM51010 (6 mg/kg s.c.) one time daily for 14 d, and antinociceptive activity was measured daily as described in *Materials and Methods*. *N* represents peak antinociception calculated from data in *L* and *M*. *O* represents AUC calculated from data in *L* and *M*. Results are mean ± SE (n = 3–15 mice per group). $^*P < 0.05$; $^{**}P < 0.01$ as determined by ANOVA followed by multiple comparison tests (Student Newman–Keuls tests) or unpaired t tests. %MPE, maximum possible effect.

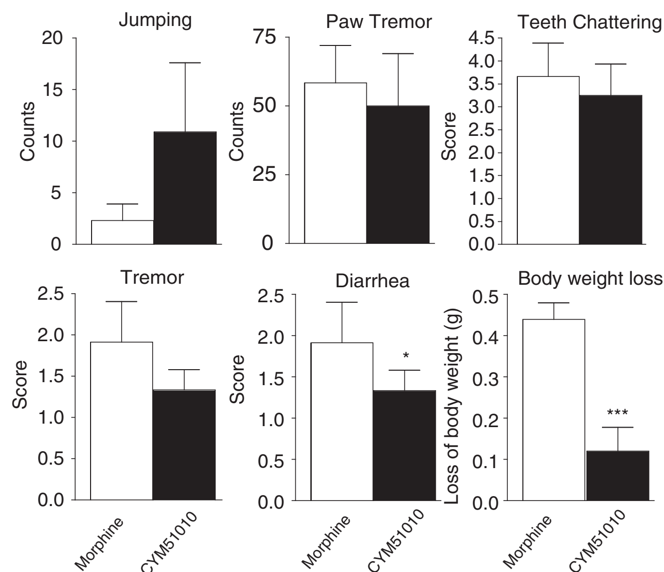

Fig. S5. Naloxone precipitated withdrawal after chronic administration of CYM51010. Mice were treated with morphine or CYM51010 (10 mg/kg s.c.) one time per day for 9 d. On the ninth day, mice were administered naloxone (5 mg/kg i.p.) 2 h after the last drug administration. The numbers of jumps, paw tremors, teeth chattering, tremors, diarrhea, and body weight loss as withdrawal signs were counted for 30 min. The results represent mean ± SE of withdrawal signs from seven separate animals. $^*P < 0.05$ vs. morphine (unpaired t test).

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)