Materials and Methods

Cell culture and transfection

HEK293 and SK-N-SH cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. CHO cells were grown in F12 media containing 10% FBS and 1% penicillin-streptomycin. HEK293 cells were transfected with either μ and δ opioid receptors, μ and α_{2A} adrenergic receptors, μ and CB1 cannabinoid receptors, δ and α_{2A} receptors, δ and CB1 receptors, or chimeric constructs to μ and δ opioid receptors; CHO cells were transfected with HA-tagged μ and Flag-tagged δ opioid receptors. CHO cells stably expressing Flag-tagged μ and Myc-tagged δ opioid receptors were generated as previously described (1). These cells were transfected with a chimeric G_{16}/G_{i3} to assess intracellular Ca^{2+} concentrations. All transfections were carried out with Lipofectamine as per manufacturer's protocol (Invitrogen).

Subtractive immunization

For induction of tolerance to immunogenic epitopes in HEK293 membranes, female balb/c mice (6-8 weeks old, 25-35 g body weight) were injected intraperitoneally (i.p.) with 5 mg HEK293 membranes and 15 min later with cyclophosphamide (100 mg/kg body weight, i.p.). The cyclophosphamide injection was repeated after 24 and 48 hours respectively. Mice were bled every 15 days and antibody titers checked by ELISA against HEK293 membranes. This protocol was repeated at 2 week intervals until stable background titers were obtained with HEK293 membranes. Mice were then given an i.p.

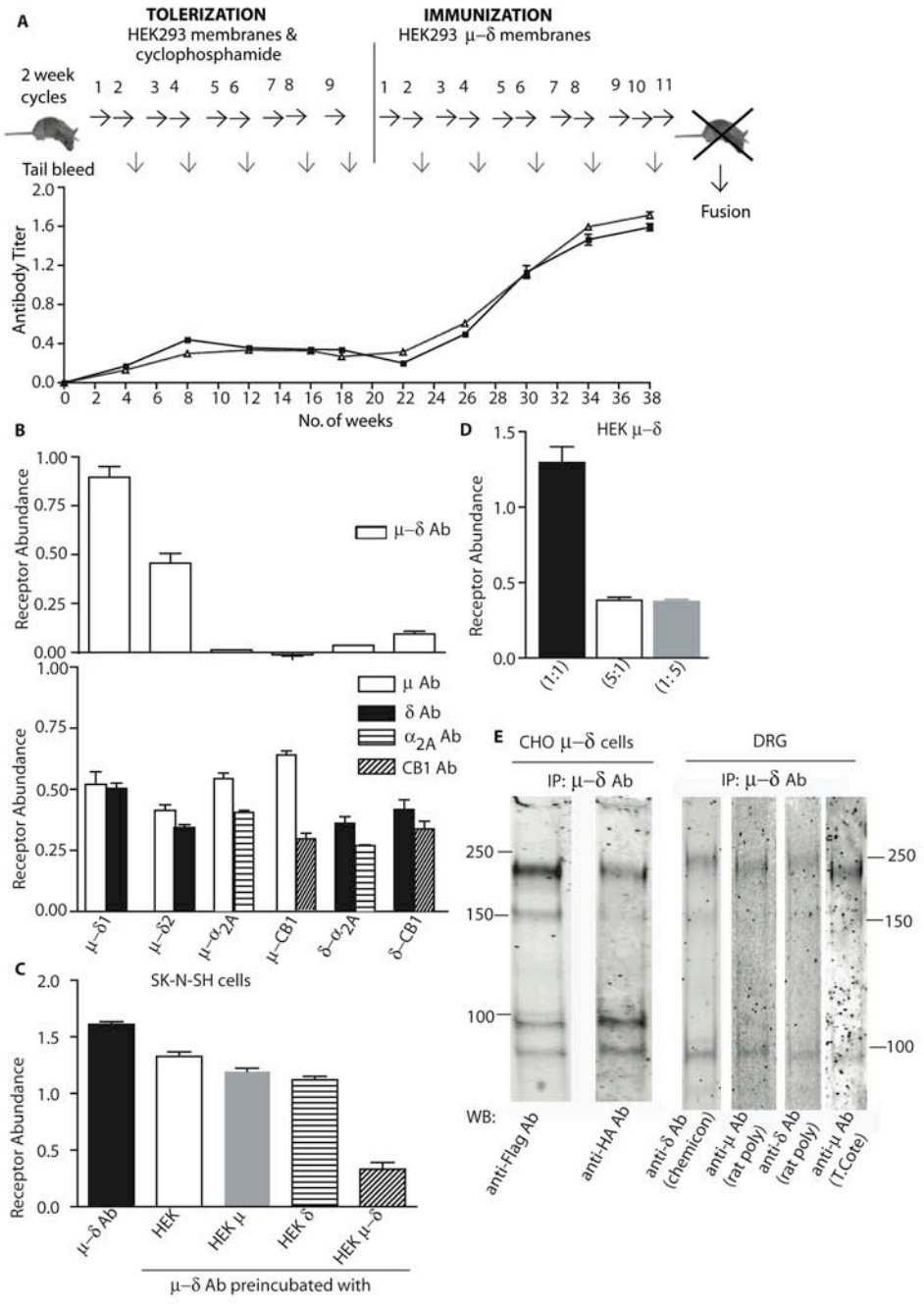
injection of membranes from HEK293 cells coexpressing μ-δ receptors (5 mg) in complete Freund's adjuvant. Booster i.p. injections of HEK293 membranes coexpressing μ-δ receptors were administered every 15 days. Antibody titers were checked by ELISA against HEK293 membranes from untransfected cells and from cells coexpressing μ-δ receptors. Spleens from animals giving a high titer with HEK293 membranes coexpressing μ-δ receptors were fused with SP-20 myeloma cells to generate monoclonal antibodies as described (2). Clones secreting monoclonal antibodies were screened by ELISA against untransfected HEK293 membranes, and HEK293 membranes expressing μ , δ or coexpressing μ-δ receptors as described (3) using 1:10 hybridoma supernatant and 1:500 horseradish peroxidase labeled anti-mouse IgG. Hybridoma supernatant from positive clones was concentrated using Centricon 10 and stored at a concentration of 10 μg protein/μl. Antibody specificity for the μ–δ heteromer was determined by ELISA with antibodies that were preincubated with membranes from HEK293 cells, or HEK293 cells expressing μ, δ, or μ and δ receptors. Membranes (10 μg) were incubated with 1:10 dilution of hybridoma supernatant in the presence of protease inhibitor cocktail (Sigma) for 3 hours at 4°C followed by centrifugation at 15,000 x g for 20 min and the supernatant was used for the ELISA assay using cells coexpressing μ and δ receptors.

Generation of polyclonal antibodies

Polyclonal antibodies to μ opioid, δ opioid, α_{2A} adrenergic and CB1 cannabinoid receptors were generated as described previously (3).

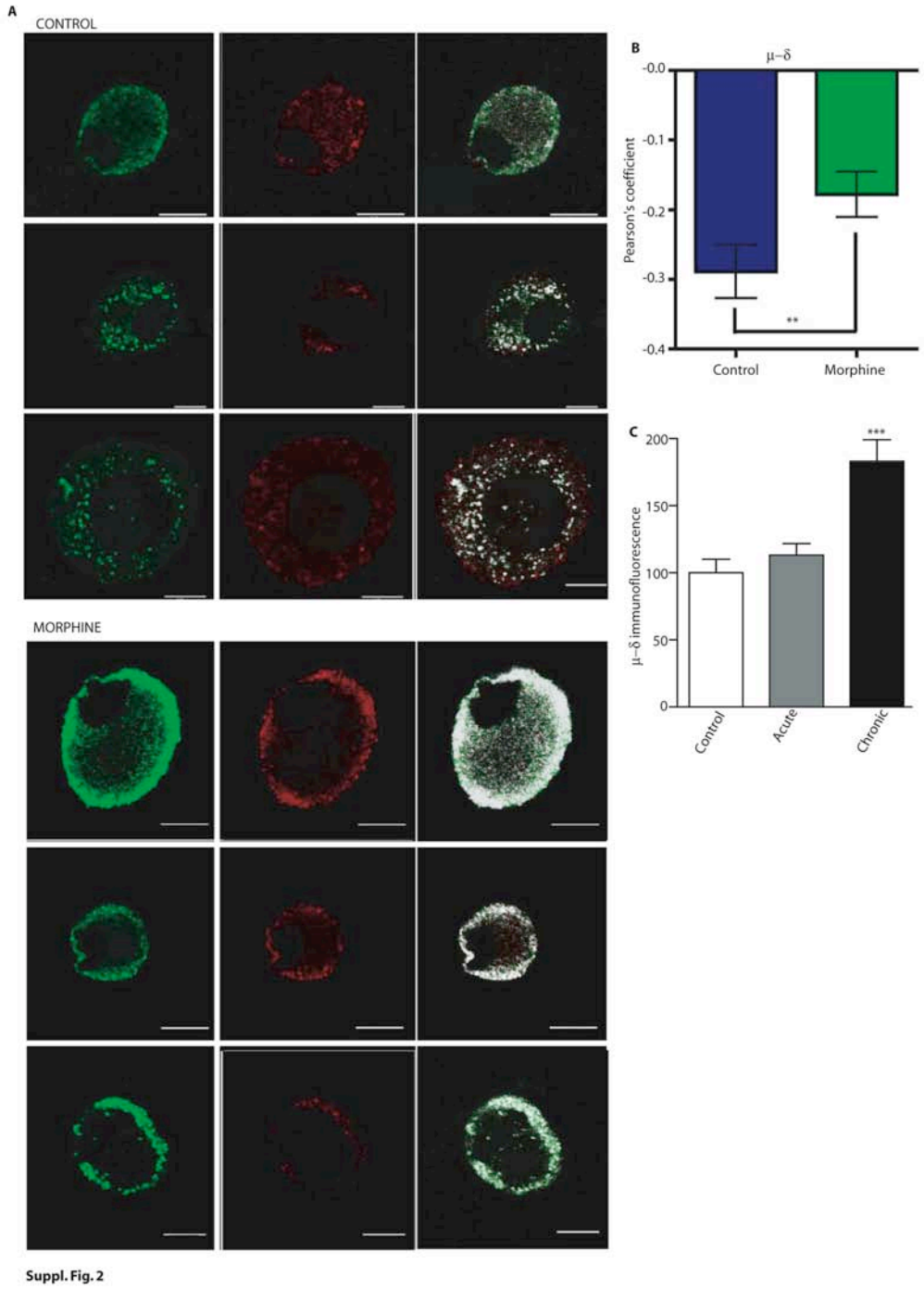
Membrane preparation

Membranes were prepared as described previously (4) from HEK293 cells (alone or expressing μ, δ, or μ and δ receptors), the cortex of wild-type (WT), μ KO, δ KO, or μ-δ KO mouse brain, from different brain regions of rats injected subcutaneously with morphine (5mg/kg), NTB (0.1mg/kg), or saline for 9 days, or from mice treated either acutely (single injection, 10mg/kg) or chronically (intermittent escalating dose protocol, 10mg/kg on day 1 to 100 mg/kg on day 5).


A
TOLERIZATION
HEK293 membranes & cyclophosphamide
IMMUNIZATION
HEK293 μ–δ membranes
2 week cycles
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11
Tail bleed
Fusion
Antibody Titer
No. of weeks
B
Receptor Abundance
μ–δ Ab
μ Ab
δ Ab
α2A Ab
CB1 Ab
μ–δ1
μ–δ2
μ–α2A
μ–CB1
δ–α2A
δ–CB1
C
SK-N-SH cells
μ–δ Ab
HEK
HEK μ
HEK δ
HEK μ–δ
μ–δ Ab preincubated with
D
HEK μ–δ
(1:1)
(5:1)
(1:5)
E
CHO μ–δ cells
DRG
IP: μ–δ Ab
WB:
anti-Flag Ab
anti-HA Ab
anti-δ Ab (chemicon)
anti-μ Ab (rat poly)
anti-δ Ab (rat poly)
anti-μ Ab (T.Cote)

Supl. Fig.1

Fig. S1. Generation and characterization of μ–δ heteromer-selective antibodies (**A**) Subtractive immunization scheme. Balb/c mice were made tolerant to immunogenic epitopes by administration of HEK293 membranes and 100 mg/kg cyclophosphamide. Mice were immunized with membranes coexpressing μ and δ receptors and monoclonal antibodies were generated. Mean antibody titers ± SEM for each individual mouse is presented. (**B**) Cells coexpressing either μ and δ , μ and α_{2A}, μ and CB1, δ and α_{2A}, or δ and CB1 receptors were subjected to ELISA using μ–δ heteromer-selective antibodies. Abundance of individual receptors was determined in parallel by ELISA with receptor-specific polyclonal antibodies. (**C**) The abundance of endogenous μ-δ heteromers in SK-N-SH cells was determined by ELISA using μ–δ heteromer-selective antibodies preincubated without or with membranes from HEK293 cells alone or HEK293 cells expressing μ, δ, or μ–δ receptors. (**D**) μ–δ heteromer-selectivity was determined by ELISA with HEK293 cells expressing μ and δ receptors at 1:1, 5:1, and 1:5 ratios. Results in (B-E) are means ± SEM (n=3-4 experiments). (**E**) μ–δ heteromers from CHO cells coexpressing HA-tagged μ and Flag-tagged δ receptors or from DRGs endogenously expressing μ and δ receptors were immunoprecipitated with the μ–δ heteromer-selective antibody and subjected to Western blot analysis with polyclonal antibodies to the epitope tags (CHO cells) or to the individual receptors (DRGs). A representative blot of 3 is shown.



Suppl. Fig. 2

Fig. S2. Subcellular distribution and colocalization of μ and δ opioid receptors. (**A**) DRG neurons were isolated from adult rats and grown in culture for 4 days prior to

experimentation. DRG neurons were treated with either vehicle (saline) or morphine (10 μM, 48 hours) before fixation and immunocytochemical labeling for μ (green) and δ (red) receptors. Image overlay illustrated in white maps shows that opioid receptor colocalization is increased after prolonged morphine treatment. (**B**) Colocalization was quantified using Pearson's colocalization coefficient. μ–δ colocalization was significantly increased in morphine-treated neurons compared to vehicle controls (mean O.D. values for vehicle: 24.3±3.96 (n=15) and for chronic morphine: 39.6±4.85 (n=18); p=0.0241)-. Data shown is representative of 4 independent experiments. (**C**) DRG neurons were treated with either vehicle (saline) or morphine (1 μM, 30 min or 48 hours) and μ–δ heteromer abundance was quantified by ELISA using μ–δ receptor selective antibodies. Results are means ± SEM (n=3 experiments). Scale bars = 10 μm. ***p<0.001.

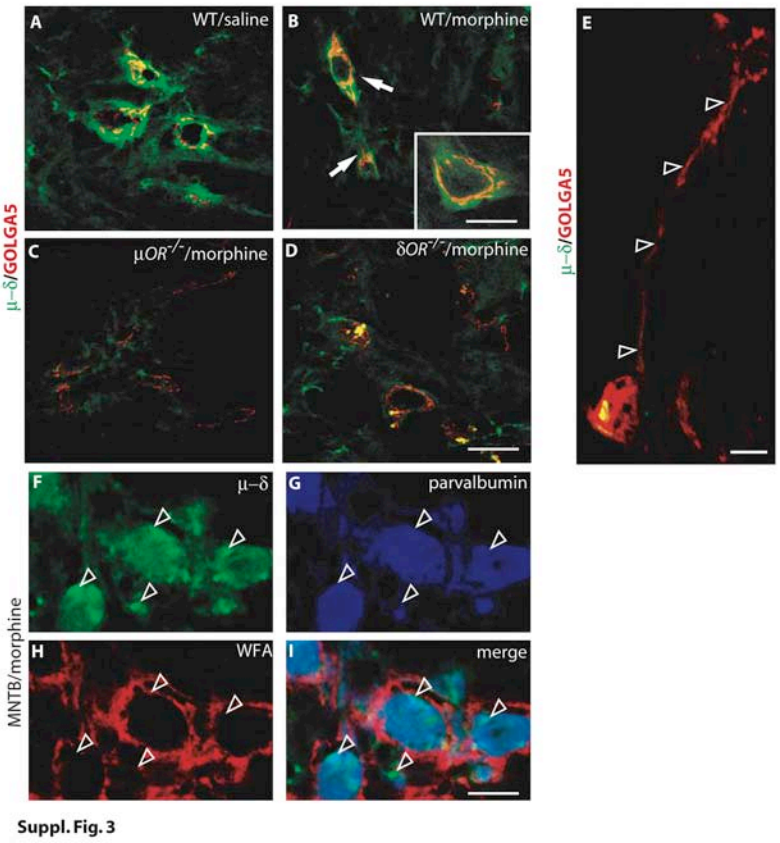


Fig. S3. Chronic morphine treatment induces increased μ–δ heteromer immunoreactivity in the brainstem. (**A-D**) A fine meshwork of μ–δ heteromer-stained small-caliber processes is visible in the absence of morphine treatment in the RVM. Perisomatic μ–δ immunoreactivity increased after morphine challenge (arrows, inset) in wild-type, but not knock-out mice. Scale bar = 18 μm. (**E**) μ–δ immunoreactivity along a putative axon (arrowheads) of an RVM neuron. Scale bar = 10 μm. (**F-I**) Subsets of MNTB neurons containing components of perineuronal nets [as detected by *Wisteria floribunda* agglutinin (WFA)] and parvalbumin positive (arrowheads) putative glycinergic cells (5) showed μ–δ heteromer immunoreactivity.

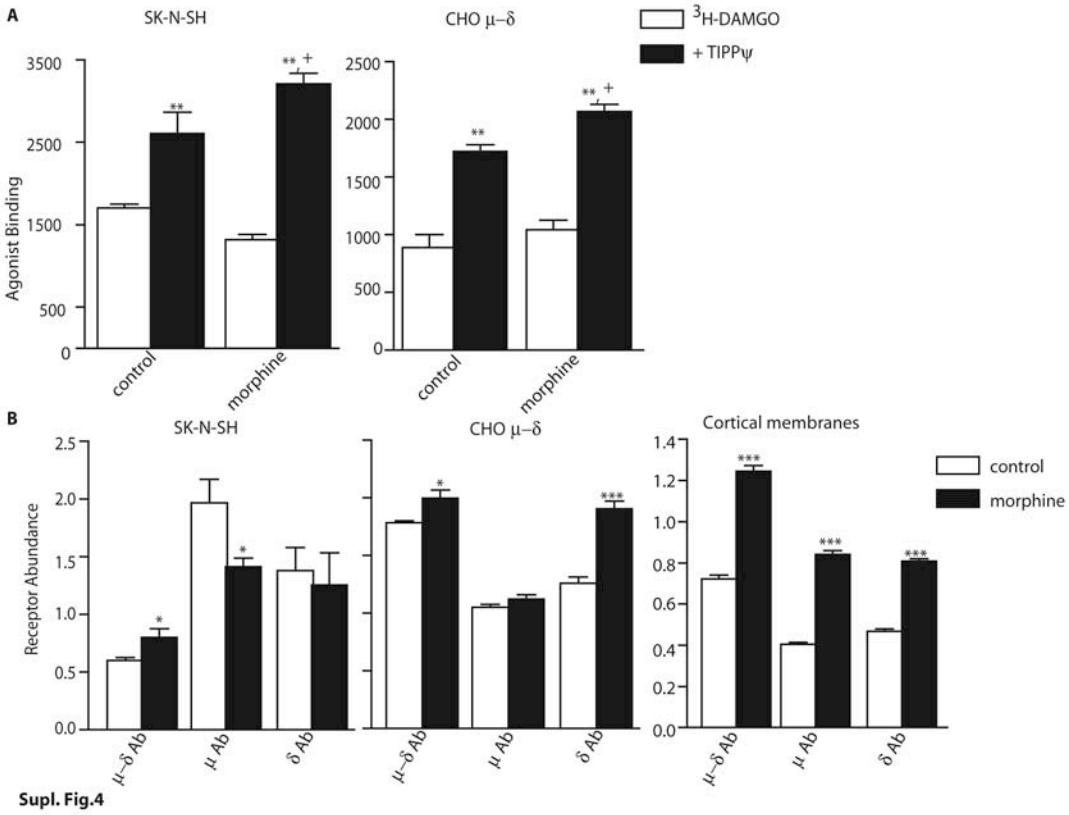
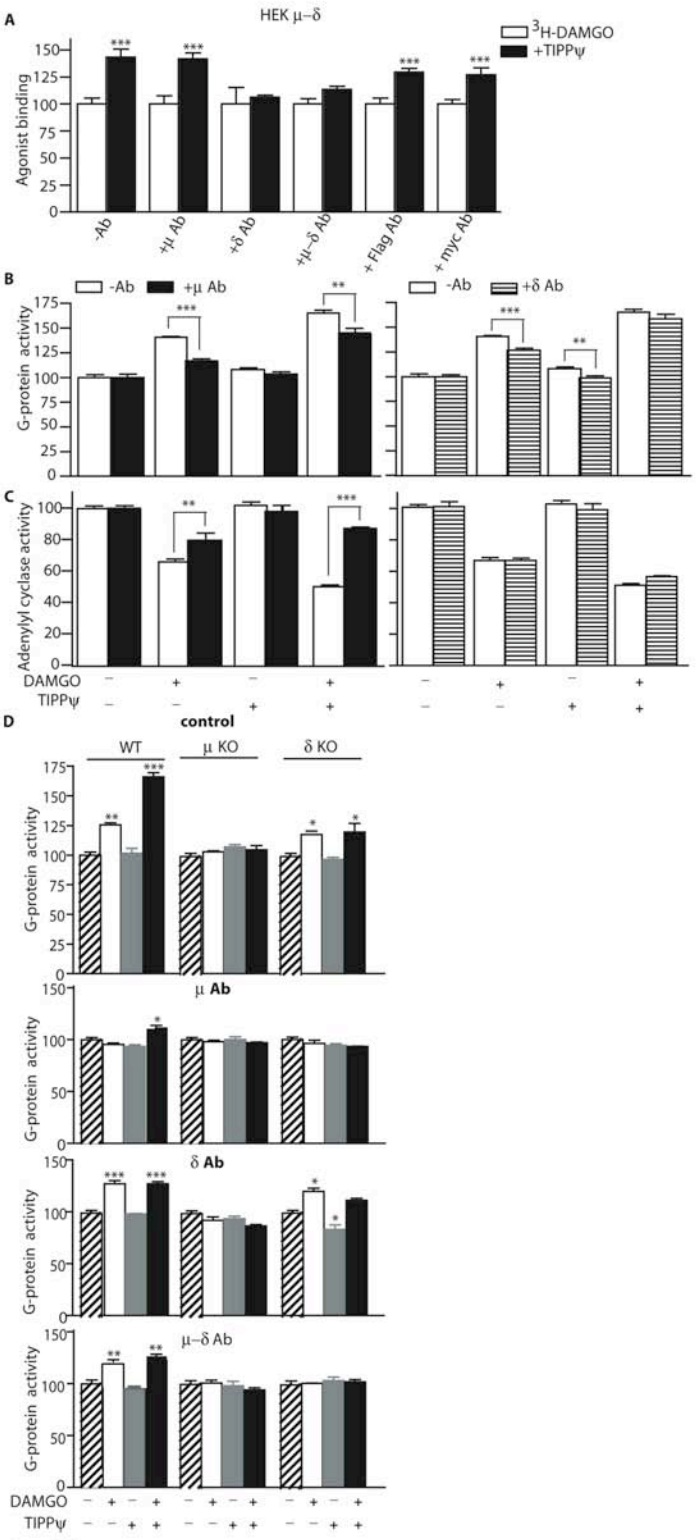


Fig. S4. Chronic morphine treatment increases μ–δ heteromer abundance. (**A**) SK-N-SH cells or CHO cells stably expressing μ and δ receptors were treated without or with morphine (1 μM) for 48 hours. Binding assays were carried using [^{3}H]DAMGO (10 nM) in the absence or presence of TIPPψ (10 nM). Results are means ± SEM (n=3 experiments). *p<0.05; **p<0.01; + p<0.05 (**B**) SK-N-SH cells or CHO cells stably expressing μ and δ receptors were treated with saline or morphine (1 μM) for 48 hours. Cortical membranes were prepared from mice treated with saline or an escalating dose of morphine. The abundance of μ, δ or μ–δ receptors in cells or membranes was quantified by ELISA with μ, δ, or μ–δ receptor selective antibodies. Results are means ± SEM (n=3

experiments). *p<0.05; ***p<0.001.


A
HEK μ–δ
^{3}H-DAMGO
+TIPPψ
Agonist binding
-Ab
+μ Ab
+δ Ab
+μ–δ Ab
+ Flag Ab
+ myc Ab
B
-Ab
+μ Ab
-Ab
+δ Ab
G-protein activity
C
Adenylyl cyclase activity
DAMGO
TIPPψ
control
D
WT
μ KO
δ KO
G-protein activity
μ Ab
δ Ab
μ–δ Ab
DAMGO
TIPPψ

Suppl. Fig 5

Fig. S5. μ–δ heteromer selective antibodies block heteromer-mediated binding and signaling. (**A**) HEK293 cells coexpressing Flag-tagged μ and Myc-tagged δ opioid receptors were pre-treated with either antibodies against μ, δ, the μ–δ heteromer, Flag, or Myc epitopes. Binding assays were carried in the presence of the antibodies using the μ receptor agonist [^{3}H]DAMGO (10 nM) in the absence or presence of the δ receptor antagonist TIPPψ (10 nM). Results are means ± SEM (n=3 experiments). (**B** and **C**) Mouse cortical membranes were preincubated without or with μ or δ antibodies and the extent of [^{35}S]GTPγS binding (expressed as G-protein activity) (C) or adenylyl cyclase activity (D), in the presence of the antibodies, in response to treatment with 1 μM DAMGO in the absence or presence of 10 nM TIPPψ was determined. Results are means ± SEM (n=3 experiments). (**D**) Cortical membranes from wild-type (WT), μ KO, or δ KO mice were preincubated without or with either μ, δ or μ–δ heteromer antibodies. Membranes were then subjected to a [^{35}S]GTPγS binding assay with 1 μM DAMGO in the absence or presence of 10 nM TIPPψ. Results are means ± SEM (n=3 experiments). *p<0.05; **p<0.01; ***p<0.001.

Table S1. Characterization of select hybridoma clones.

Clone No.	O.D. 490 nm			
	HEK293	HEK293 μ	HEK293 δ	HEK293 μ–δ
1A4	0.13 ± 0.004	0.68 ± 0.04	0.04 ± 0.008	0.65 ± 0.03
2B1	0.15 ± 0.007	0.051 ±0.02	0.65± 0.032	0.63 ± 0.03
1E12D1	0.10 ± 0.007	0.21 ± 0.01	0.22 ± 0.013	1.80 ± 0.04

Membranes from untransfected HEK293 cells or cells expressing μ, δ, or μ and δ receptors were plated on 96-well poly-L-lysine coated plates and subjected to ELISA by incubation with 1:10 hybridoma supernatant and 1:500 horseradish peroxidase labeled anti-mouse IgG. The receptor recognition was measured using *o*-phenylenediamine as substrate. Results are mean ± SEM (n=3 experiments).

1A4, μ monoclonal Ab; 2B1, δ monoclonal Ab; 1E12D1, μ-δ monoclonal Ab.

Table S2. Heteromer-selective antibody recognition using chimeric μ-δ constructs

μ-δ chimeras		% Ab recognition
μ/δ		100 ± 7.7
$\mu_N\delta_C/\mu_N\delta_C$		16 ± 3.4***
$\mu_N\delta_C/\mu$		9 ± 1.5***
$\mu_N\delta_C/\delta$		13 ± 2.1***
$\mu_N\delta_C/\delta_N\mu_C$		64 ± 5.1***
$\delta_N\mu_C/\delta_N\mu_C$		3 ± 4.5***
$\delta_N\mu_C/\mu$		9 ± 2.9***
$\delta_N\mu_C/\delta$		14 ± 1.3***
$\delta_N\mu_C/\mu_N\delta_C$		61 ± 1.5***

HEK293 cells were cotransfected with full length wild-type μ and δ receptor cDNAs or with a combination of chimeric μ-δ constructs. The degree of receptor recognition was determined by ELISA. Results are means ± SE (n=3 experiments) . ***p<0.001 compared to μ/δ, Dunnett's test.

References

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