Supporting Information

Yekkirala et al. 10.1073/pnas.1016277108

SI Materials and Methods

Synthesis of 17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(2′-naphthyl)carboxamido]morphinan (NNTA). β-Naltrexamine (400 mg, 1.16 mmol), naphthoic acid (399 mg, 2.32 mmol), and benzotriazole-1-yl-oxy-Tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (1.03 g, 2.32 mmol) were dissolved in methylene chloride (15 mL). To this solution, *N, N*-Diisopropylethylamine (DIPEA) (600 mL, 3.25 mmol) was added and the mixture was stirred at room temperature for 16 h. The solution was concentrated under reduced pressure and the residue was taken up in MeOH (15 mL), and K_2CO_3 was added (1.2 g). After 1 h at room temperature, the mixture was concentrated to dryness. The residue was purified by silica gel chromatography (eluted with EtOAc/Hexanes: 4/1) to afford the desired product (NNTA) as a white solid. NNTA was recrystallized from an acetone/hexanes (1/4: vol/vol) mixture (473 mg, 82%). NNTA was subsequently converted into the HCl salt for biological testing.

The melting point was determined on a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra and ^{13}C NMR spectra were determined on a Bruker 400 MHz instrument and calibrated using an internal reference. ESI mode mass spectra were recorded on a BrukerBioTOF II mass spectrometer. Elemental analyses were performed by M-H-W Laboratories.

Base Form. ^{1}H NMR (DMSO-d_6) δ: 0.12 (m, 2H) ; 0.47 (m, 2H) ; 0.86 (m, ^{1}H) ; 1.27–1.63 (m, ^{4}H) ; 1.85–2.03 (m, ^{2}H) ; 2.09 (m, ^{1}H) ; 2.22 (m, ^{2}H) ; 2.37 (m, ^{2}H) ; 3.01 (m, ^{2}H) ; 3.75 (m, ^{1}H) ; 4.76 (d, ^{1}H, $J_{H5\text{-}H6}$ = 7.8 Hz) ; 4.92 (bs, ^{1}H, O$\underline{H}$-14) ; 6.54 (d, ^{1}H, $J^1_{H\text{-}H2}$ = 8.1 Hz) ; 6.60 (d, ^{1}H, $J_{H2\text{-}^1H}$ = 8.1 Hz) ; 7.59–7.62 (m, ^{2}H) ; 7.96–8.03 (m, ^{4}H) ; 8.50 (s, ^{1}H), 8.84 (d, ^{1}H amide, $J_{N\underline{H}\text{-}H6}$ = 8.2 Hz) ; 9.05 (bs, ^{1}H, O$\underline{H}$-3) ; ^{13}C NMR (DMSO-d_6) δ: 3.52, 3.65, 9.21, 22.12, 24.69, 30.06, 30.27, 43.66, 47.01, 51.63, 58.35, 61.71, 69.58, 90.64, 116.93, 118.36, 123.45, 124.08, 126.68, 127.43, 127.55 (×2), 127.77, 128.80, 131.36, 131.58, 132.08, 134.05, 140.41, 142.06, 165.52 ; mp = 199–201 °C. Anal. Calcd. for $C_{31}H_{32}N_2O_4$: C,74.98 ; H, 6.50 ; N, 5.64. Found: C, 73.85 ; H, 6.13 ; N, 5.52. ESI-TOF MS *m/z*: 497.2930 (MH^+), 993.5777 ($2xMH^+$).

Salt Form. ^{1}H NMR salt form (DMSO-d_6) δ: 0.53–0.68 (m, ^{4}H) ; 1.08 (m, ^{1}H) ; 1.46 (m, ^{2}H) ; 1.63 (m, ^{1}H) ; 1.79–1.95 (m, ^{2}H) ; 2.48 (m, ^{2}H) ; 2,87 (m, ^{1}H) ; 3.03–3.12 (m, ^{2}H) ; 3.35 (m, ^{2}H) ; 3.75 (m, ^{1}H) ; 3.90 (m, ^{1}H) ; 4.90 (d, ^{1}H, $J_{H5\text{-}H6}$ = 7.8 Hz) ; 6.29 (bs, ^{1}H, O$\underline{H}$-14) ; 6.65 (d, ^{1}H, $J^1_{H\text{-}H2}$ = 8.1 Hz) ; 6.74 (d, ^{1}H, $J_{H2\text{-}^1H}$ = 8.1 Hz) ; 7.59–7.62 (m, ^{2}H) ; 7.97–8.03 (m, ^{4}H) ; 8.52 (s, ^{1}H), 8.90 (d, ^{1}H amide, $J_{N\underline{H}\text{-}H6}$ = 8.2 Hz) ; 9.38 (bs, ^{1}H, O$\underline{H}$-3) ; ^{13}C NMR salt form (DMSO-d_6) δ: 2.57, 5.10, 5.70, 22.99, 23.80, 27.30, 29.32, 40.07, 45.56, 46.45, 51.23, 56.59, 61.56, 69.69, 89.86, 117.83, 119.23, 120.59, 124.05, 126.71, 127.49, 127.57 (×2), 127.81, 128.80, 129.66, 131.44, 132.07, 134.09, 141.28, 142.10, 165.59 mp > 260 °C. Anal. Calcd. for $C_{31}H_{33}ClN_2O_4$: C, 69.85 ; H, 6.24 ; N, 4.67. Found: C, 67.79 ; H, 6.44 ; N, 4.67. ESI-TOF MS *m/z*: 497.2930 (MH^+).

Guinea Pig Ileum. Longitudinal muscle strips were prepared for experiments using the method of Rang (1) and assays were conducted as described previously (2).

Competition Binding. Experiments were performed using HEK-293 cells genetically modified to produce wild-type human μ-, κ-, or δ-opioid receptors and coexpressing μ/κ-opioid receptors. Ten concentrations of the tested compounds (50 μL) were added to test tubes containing 0.5 nM [^{3}H]diprenorphine (~ 1.0 × K_D) (50 μL) or selective radioligands [^{3}H]DAMGO and [^{3}H]U69593 (both 2.0 nM) and whole cells (75 mm^2 plate, 80–90% confluent) suspended in 12 mL Hepes buffer (25 mM, pH = 7.4) (400 μL). Final volume was 500 μL. Nonspecific binding was measured in the presence of 10 μM naloxone. Assays were incubated at room temperature for 90 min and then filtered using a Brandel M-48 tissue harvester through Whatman GF/C filter paper that was presoaked in 0.25% poly(ethyleniminie). Filters were washed three times with ice-cold Hepes buffer (see above), and the radioactivity counted using a LS 6500 liquid scintillation counter (Beckman). All measurements were performed in triplicate. IC_{50} values were calculated using Prism software (GraphPad) using nonlinear regression of the data normalized to fit a sigmoidal dose–response curve with a variable slope (100% defined at concentration = 0 (total binding) and 0% defined at the value of nonspecific binding). K_i values were determined from the Cheng–Prusoff equation assuming a single-site binding model. Values reported are mean K_i ± SEM of three or more independent experiments (3, 4).

[^{35}S]GTPγS Assay. The assay was set up as described previously (5, 6). Briefly, membranes from μ-, κ-, and μ/κ-cells were incubated with varying concentrations of NNTA and [^{35}S]GTPγS (Perkin-Elmer) in a 96-well plate. The incubation was performed at 37 °C for 1 h in membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 0.5% BSA), filtered and counted for radioactivity. Three replications were performed for each treatment.

Immunocytochemistry. Two-color immunofluorescence was used to analyze coexpression of μ- and κ-opioid receptors as previously described (5). Briefly, HEK-293 cells coexpressing HA-μ and FL-κ were incubated with goat anti-HA (Abcam; ab9134) antibody and rabbit anti-FLAG (Abcam; 1162) antibody at a final working concentration of 1:100 for 60 min at 4 °C. After rinsing three times with 50 mM PBS (pH 7.2), cells were fixed with 2% formaldehyde for 10 min at room temperature. Then cells were washed (3 × 15 min) with PBS and incubated at room temperature with the mixture of antigoat NL-493 (NL003; R&D Systems) and antirabbit NL-557 (NL004; R&D Systems) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 × 15 min) and mounted under coverslips with antifade mounting media iBright Plus (SF40000-10; Neuromics) containing DAPI. An Olympus FluoView1000 confocal microscope was used for image collection.

Animals Protocols. For studies performed at the University of Minnesota, male ICR-CD1 mice (18–25 g or 30–35 g; Harlan), were housed in groups of eight in a temperature- and humidity-controlled environment with unlimited access to food and water and maintained on a 12 h light/dark cycle. The μ-opioid receptor knockout mice (BALB/C X C57BL/6 MORKO) were a generous gift from Dr. Sabita Roy and have been described previously (7). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

For studies performed at the Louisiana State University Health Sciences Center in Shreveport (LSUHSC-S), male ICR mice weighing 25–35 g (Harlan Sprague–Dawley) were used in the i.v. and chronic i.c.v. studies. The mice were housed in Association for Assessment and Accreditation of Laboratory Animals-ac-

credited animal facilities with 12 h light/dark cycles and food and water available ad libitum. All procedures were approved by the IACUC at LSUHSC-S.

Antinociceptive Studies. All solutions were dissolved in distilled water. Controls with only distilled water showed no antinociception. All ligands were administered in a 5-μL volume in conscious mice according to the methods of Hylden and Wilcox (8) for i.t. and Haley and McCormick (9) for i.c.v. injections.

Quantal Method. For measuring the antinociception of NNTA in mice, the drugs were administered so that the antagonist and agonist effects would peak simultaneously. The peak times for the antagonists were as follows: β-FNA and norBNI were both 20 min. The agonist peak times for NNTA, i.t. 5 min and i.c.v. 10 min. Antinociception was measured using the modified radiant heat tail-flick test (10). Briefly, a radiant heat source was applied to the dorsal side of the tail, and the latency to flick away from the heat source was recorded. The data were made quantal by designating a positive antinociceptive response of an animal as those that increased their latency to tail flick (after drug treatment) by at least three SD above the mean of the baseline latency of the whole group (11). The light source was manually turned off if the mouse did not flick its tail after the three SD criteria for a positive response. At least three groups of 8–10 mice were used for each drug paradigm, and each mouse was used only once. ED_{50} values and 95% confidence intervals (CI) were calculated by using the parallel line assay (12). When ED_{50} values were compared, all of the data were analyzed together and values were considered significantly different if they did not lie in each other's 95% confidence limits at $P < 0.05$.

Percent Maximal Possible Effect (%MPE) Method. The tail-flick assay was performed as described by D'Amour and Smith (13) and modified by Dewey et al. (14). For the measurement the latency of the tail-flick measurement, the mice were held gently in one hand with the tail positioned in the apparatus (Tail-Flick Analgesia Meter; Columbus Instruments) for radiant heat stimulus. The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat was set at setting 8 so that the animal flicked its tail within 2–3 s. The test latency was measured once before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10-s maximum cutoff time was used to prevent damage to the tail. Antinociception was quantified according to the method of Harris and Pierson (15) as the %MPE, which is calculated as: %MPE = (Test – Control/10 – Control) × 100. At least three groups of 8–10 mice were used for each dose–response curve, and each mouse was used only once. ED_{50} values with 95% CI were computed with GraphPad Prism 4 by using nonlinear regression methods.

Intravenous and Chronic i.c.v. Studies. For the i.v. and chronic i.c.v. studies, mice were tested using the %MPE radiant heat tail-flick test as described above. For all experiments, baseline latency times were determined, drugs were injected either i.v. or i.c.v., and tail-flick latency times were again determined at the time of peak antinociceptive response as determined from preliminary studies. For morphine i.v., the time of peak antinociception was 15 min, for i.v. NNTA, 20 min, and for i.c.v. NNTA, 10 min. Animals were killed at the end of the experiment and each animal was used only once, for one dose. The ED_{50} (95% confidence interval) values were calculated using Prism software.

Tail-Flick Assay for Intrathecal Tolerance. For measuring intrathecal tolerance, animals were injected according to a modified methodology of Fairbanks et al. (16). The mice were injected twice on day 1 with the 80–90 MPE% dose (250 pmol per mouse) with a 5-h gap between injections. The same regimen was repeated again on day 2. On the third day, the mice were injected once with an acute dose of NNTA (250 pmol per mouse) following which, tail-flick latencies were measured and %MPE values were calculated to look for tolerance.

Chronic Infusion. Mice were infused i.c.v. with saline or NNTA (7.1 nmol) for 3 d using osmotic minipumps as previously described (17). On the fourth day, animals were injected with 1 mg/kg naloxone (s.c.), placed in a 4-L glass beaker, and number of jumps in 10 min was recorded. After testing, the minipumps were removed and after 2 h, the animals were administered an acute dose of NNTA i.c.v. Tail-flick latencies were measured and %MPE values were calculated as described above.

Conditioned Place Preference (CPP). Mice were tested for CPP as previously described (18). Drugs were administered i.v. for 3 d of conditioning. On the fourth day, time spent on the drug-paired or saline-paired side was recorded. Doses of morphine and naloxone used were as previously described (18). Data were analyzed using Student's *t* test.

1. Rang HP (1964) Stimulant actions of volatile anaesthetics on smooth muscle. *Br Pharmacol Chemother* 22:356–365.
2. Portoghese PS, Takemori AE (1985) TENA, a selective kappa opioid receptor antagonist. *Life Sci* 36:801–805.
3. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Rose J (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
4. Werling LL, Zarr GD, Brown SR, Cox BM (1985) Opioid binding to rat and guinea-pig neural membranes in the presence of physiological cations at 37 degrees C. *J Pharmacol Exp Ther* 233:722–728.
5. Yekkirala AS, Kalyuznhy AE, Portoghese PS (2010) Standard opioid agonists activate heteromeric opioid receptors: evidence for morphine and [d-Ala2-MePhe4-Glyol5] enkephalin as selective μ–δ agonists. *ACS Chem Neurosci* 1:146–154.
6. Mullaney I (1999) G proteins and their identification. *Signal Transduction: A Practical Approach*, ed Milligan G (Oxford Univ Press, London), 2nd Ed, pp 100–101.
7. Roy S, Barke RA, Loh HH (1998) MU-opioid receptor-knockout mice: Role of mu-opioid receptor in morphine mediated immune functions. *Brain Res Mol Brain Res* 61: 190–194.
8. Hylden JLK, Wilcox GL (1980) Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. *Eur J Pharmacol* 67:313–316.
9. Haley TJ, McCormick WG (1957) Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br Pharmacol Chemother* 12:12–15.
10. Tulunay FC, Takemori AE (1974) The increased efficacy of narcotic antagonists induced by various narcotic analgesics. *J Pharmacol Exp Ther* 190:395–400.
11. Tallarida RJ (2000) *Drug Synergism and Dose–Effect Data Analysis* (Chapman and Hall/CRC, Boca Raton, FL), pp 91–116.
12. Finney DJ (1964) *Statistical Methods in Biological Assay* (Hafner, New York), 2nd Ed.
13. D'Amour FE, Smith DL (1941) A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72:74–79.
14. Dewey WL, Harris LS, Howes JF, Nuite JA (1970) The effect of various neurohumoral modulators on the activity of morphine and the narcotic antagonists in the tail-flick and phenylquinone tests. *J Pharmacol Exp Ther* 175:435–442.
15. Harris LS, Pierson AK (1964) Some narcotic antagonists in the benzomorphan series. *J Pharmacol Exp Ther* 143:141–148.
16. Fairbanks CA, Kitto KF, Nguyen HO, Stone LS, Wilcox GL (2009) Clonidine and dexmedetomidine produce antinociceptive synergy in mouse spinal cord. *Anesthesiology* 110:638–647.
17. Li G, et al. (2009) Corrections to design, synthesis, and biological evaluation of 6α- and 6β-N-heterocyclic substituted naltrexamine derivatives as μ opioid receptor selective antagonists. *J Med Chem* 52:1416–1427.
18. Lenard NR, Daniels DJ, Portoghese PS, Roerig SC (2007) Absence of conditioned place preference or reinstatement with bivalent ligands containing opioid mu agonist and delta antagonist pharmacophores. *Eur J Pharmacol* 566:75–82.

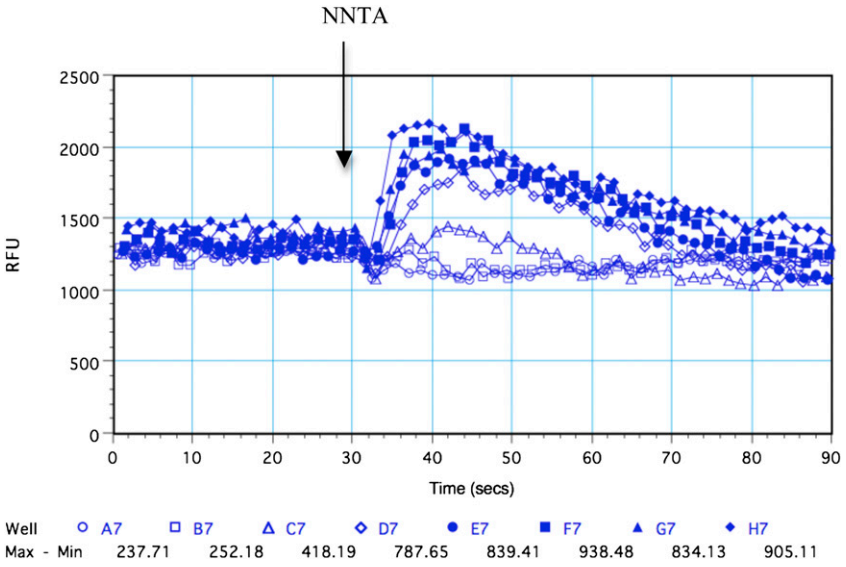

Fig. S1. Raw calcium fluxes induced by increasing concentrations of NNTA.

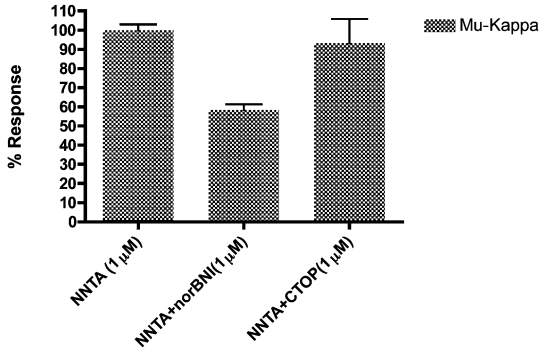

Fig. S2. NNTA is antagonized by norBNI in cells coexpressing μ- and κ-receptors. norBNI (1 μM) significantly antagonized NNTA (1 μM), whereas CTOP (1 μM) did not antagonize NNTA.

Table S1. Competition binding of [^{3}H]diprenorphine and NNTA in HEK-293 cells singly expressing opioid receptors

Cell type	K_i (pM)
μ	0.077 ± 0.003
κ	0.084 ± 0.039
δ	1390 ± 300

K_d values of [^{3}H]diprenorphine: 0.16 nM (μ), 0.12 nM (κ), and 0.36 nM (δ). Data shown as mean ± SEM (n = 3).

Table S2. Competition binding of selective radioligands and NNTA in HEK-293 cells coexpressing μ/κ-receptors or individually expressing μ- and κ-receptors

	Radioligands	
Cell type	[^{3}H]-DAMGO K_i (pM)	[^{3}H]-U69593 K_i (pM)
μ/κ-coexpressed	2.07 ± 1.18	3.39 ± 0.38
μ	1.25 ± 0.176	—
κ	—	9.47 ± 5.75

K_d values: [^{3}H]-DAMGO, 4.64 nM (μ) and 4.08 nM (μ/κ); [^{3}H]-U69593, 8.16 nM (κ) and 5.62 nM (μ/κ). Data shown as mean ± SEM (n = 3).

Table S3. EC_{50} values (nM) for Ca^{2+} release from HEK-293 cells stably expressing opioid receptors

Cells	NNTA	DAMGO	U69593	DPDPE
μ/κ	I: 1.3E-07 ± 1.1E-07 II: 0.70 ± 0.33	13.4 ± 10.8	6.5 ± 4.1	—
κ/δ	17.9 ± 10.6	—	2.8 ± 1.3	21.0 ± 20.4
μ/δ	>1,000	1.5 ± 1.4	—	17.6 ± 15.9
μ	>1,000	10.6 ± 0.4	—	—
κ	51.4 ± 11.0	—	33.7 ± 22.1	—
δ	>1,000	—	—	11.0 ± 10.5

There are two EC_{50} values for μ/κ due to the biphasic concentration curve.

Table S4. AUC_{peak}

Cells	NNTA	DAMGO	U69593	DPDPE
μ/κ	15,947 ± 1,087	5,562 ± 668	4,899 ± 566	—
κ/δ	2,462 ± 197	—	11,803 ± 1,291	10,153 ± 1,479
μ/δ	57 ± 42	12,054 ± 1,281	—	10,504 ± 969
μ	5,203 ± 1,622	9,989 ± 702	—	—
κ	5,548 ± 1,285	—	6,452 ± 737	—
δ	2,441 ± 409	—	—	8,319 ± 1,117

AUC, area under the curve (RFU x seconds); RFU, relative fluorescent units. Values presented as mean ± SEM (n = 12–16).