

Putative Kappa Opioid Heteromers As Targets for Developing Analgesics Free of Adverse Effects

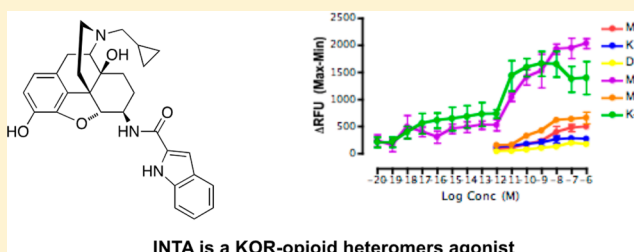
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S Supporting Information

ABSTRACT: It is now generally recognized that upon activation by an agonist, β -arrestin associates with G protein-coupled receptors and acts as a scaffold in creating a diverse signaling network that could lead to adverse effects. As an approach to reducing side effects associated with κ opioid agonists, a series of β -naltrexamides **3–10** was synthesized in an effort to selectively target putative κ opioid heteromers without recruiting β -arrestin upon activation. The most potent derivative **3** (INTA) strongly activated KOR-DOR and KOR-MOR heteromers in HEK293 cells. In vivo studies revealed **3** to produce potent antinociception, which, when taken together with antagonism data, was consistent with the activation of both heteromers. **3** was devoid of tolerance, dependence, and showed no aversive effect in the conditioned place preference assay. As immunofluorescence studies indicated no recruitment of β -arrestin2 to membranes in coexpressed KOR-DOR cells, this study suggests that targeting of specific putative heteromers has the potential to identify leads for analgesics devoid of adverse effects.

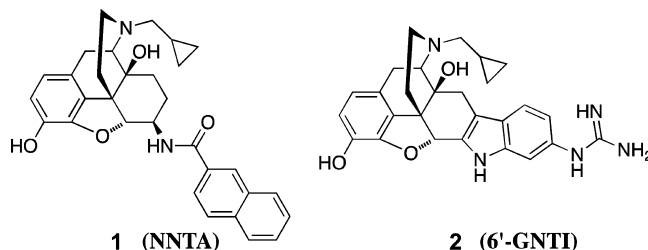


INTRODUCTION

Although homomeric G protein-coupled receptors (GPCRs) have been reported to be functional, more recent studies have suggested that many members of the GPCRs may exist as oligomeric heteromers.^{1–6} The existence of opioid heteromers has been demonstrated using a wide range of experimental techniques including coimmunoprecipitation, immunocytochemistry, bioluminescence, and Foerster resonance energy transfer (BRET and FRET, respectively).^{6,7} It is now generally recognized that following G protein activation, the β -arrestin that associates with its target can act as scaffold for binding a host of intracellular mediators that initiate other signaling pathways, thereby leading to a diverse signaling network.^{8–15} As this network may be a source of some adverse effects associated with opioid analgesics, we have considered the possibility that receptor biasing could be mediated via activation of a specific heteromer whose interaction with β -arrestin is reduced or modified.

The present study describes an approach to developing analgesics that target putative κ opioid receptor (KOR)-containing heteromers without producing the adverse effects known to be associated with this receptor. On the basis of the structural requirements of ligands (**1**,¹⁶ **2**¹⁷) that activate κ/μ opioid receptor (KOR-MOR) and κ/δ opioid receptor (KOR-DOR) heteromers, a focused library of compounds was evaluated for activating putative KOR heteromers without recruitment of β -arrestin-2. This led to the identification *N*-2'-Indolyl-naltrexamine

3 (INTA), which produces potent antinociception in mice without aversion, tolerance, or dependence.



CHEMISTRY

Given that **2**¹⁷ was reported to possess δ - κ agonist activity, the influence of an indole group on agonist selectivity was explored through a series of β -naltrexamine amides **3–10** that contain an indolyl moiety or closely related isosteric groups. These ligands were synthesized in a fashion similar to that described previously.¹⁸

BIOLOGICAL RESULTS

Intracellular Ca^{2+} Release Studies in HEK293 Cells. Initial studies involved testing target compounds **3–10** for

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Table 1. Antinociception of 3–10 in Mice

compound ^a	mode of administration	ED ₅₀ pmol/mouse (95% CI)	24 h tolerance ^b	ratio icv/i. t
INTA	i.t.	21.27 (13.90–32.54)	no	59
3	icv	1252.2 (948–1652)	no	
4	i.t.	23.06 (16–33)	no	24
	icv	550.4 (325–933)	yes	
5	i.t.	29.39 (21.91–39.43)	no	NA ^d
	icv	partial agonist ^c		
6	i.t.	partial agonist		NA
	icv	partial agonist		
7	i.t.	partial agonist		NA
	icv	partial agonist		
8	i.t.	295.0 (140.04–620.0)	yes	NA
	icv	partial agonist		
9	i.t.	partial agonist		NA
	icv	partial agonist		
10	i.t.	191.9 (133–276)	no	3
	icv	580.8 (417–808)	no	

^aPeak times for the dose response curves were as follows for i.t.: 3, 4, 8, and 10, all 10 min; 5, 6, 7, 9, all 20 min. For icv: 3, 4, and 10, 10 min. ^bAcute tolerance was calculated using the highest dose of the dose–response curve on day 1 and repeated on day 2. If there was no significant difference between the 2 days, the animals were said to be not tolerant. ^cPartial agonist is defined as when the maximum %MPE was ≤60% ^dNA: not applicable.

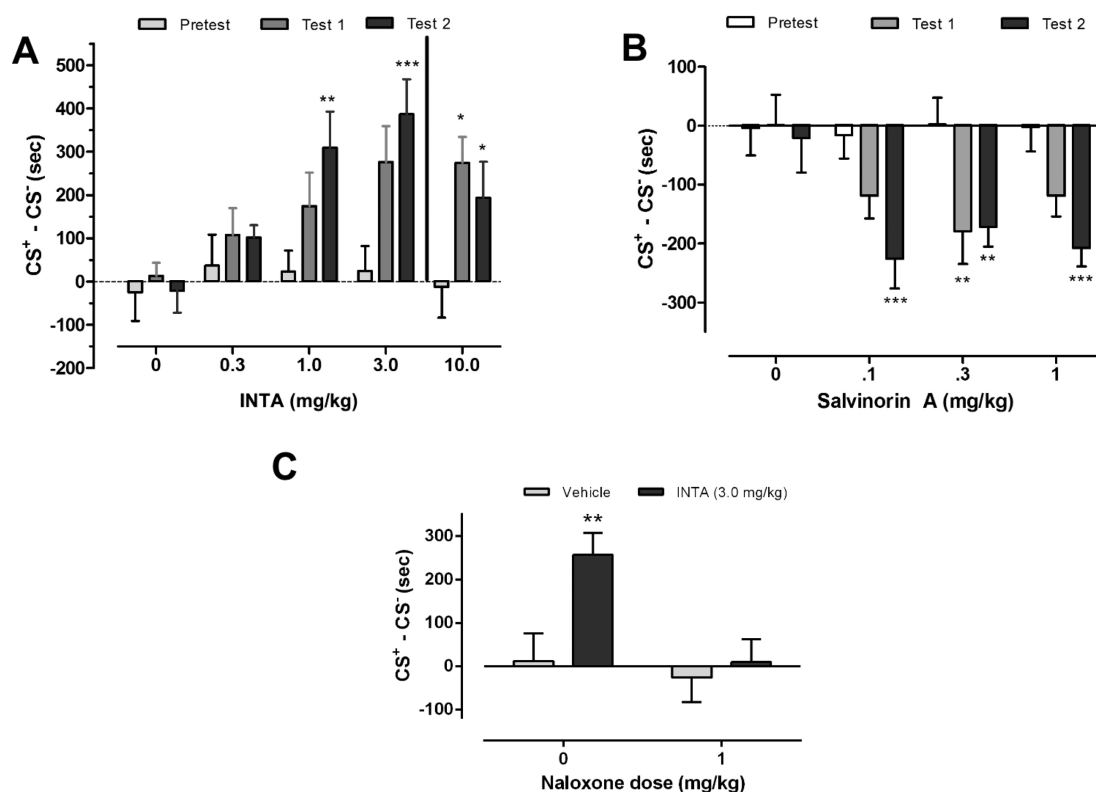


Figure 2. (A) 3 (INTA) did not produce aversion in mice. A strong dose-dependent rewarding effect was observed in a wide range of doses (0.3–10 mg/kg, sc). (B) On another hand, salvinorin A (0.3–1.0 mg/kg, sc) in the same protocol produced a strong aversion. (C) 3 condition preference was reversed by naloxone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Bonferroni t test comparison versus vehicle control (same test). Test 1 and test 2 were performed following four and eight days of conditioning, respectively.

The *N*-methyl analogue 4 had identical potency to 3 via the i.t. route, with an icv/i.t. ED₅₀ ratio approximately half that of 3. Acute tolerance was observed when 4 was administered icv. A possible reason for the discrepancy between in vivo and the cell-based data could be due to the greater lipophilicity of 4 or to targeting a different KOR heteromer. The 3'-regioisomer 5 displayed i.t. antinociception which was similar to that of 3 and

was not accompanied by 24 h tolerance. In contrast to 3, it was found to be a partial agonist when administered i.t., thereby precluding use of the naloxone-precipitated jumping assay. As the 4'-regioisomer 6 exhibited mixed agonist–antagonist activity after i.t. or icv administration, no ED₅₀ values were obtained. The 5'-chloro analogue 7 possessed a similar mixed agonist–antagonist profile, while the 5'-fluoro derivative 8 was found to

be a weak full agonist with tolerance by the i.t. route and a partial agonist when administered icv. Partial agonism by both routes of administration was also observed with benzothiaephene analogue 9. The benzofuran congener 10 behaved as a weak full agonist without tolerance by both routes of administration.

In Vivo Studies of 3 with Opioid Antagonists. Initial studies involved use of the nonselective antagonist, naloxone, via the i.t. route (Supporting Information Figure S2). Naloxone (500 nmol/mouse) produced a potent antagonism, as indicated by a 15-fold rightward shift, which is consistent with an opioid receptor-mediated mechanism. The pharmacologic selectivity of compound 3 was evaluated with selective antagonists norBNI^{22,23} (κ), NTB²⁴ (δ), and β -FNA^{25,26} (μ) used alone or in combination. Each antagonist when measured individually using the standard antagonist dose did not shift the dose–response curve of 3. To measure for synergism, a theoretical AD₅₀ was calculated based on the ratio of each antagonist to the other antagonist. The theoretical AD₅₀ was compared to the actual AD₅₀ measured. Synergism was considered significant if the 95% of the confidence intervals (CI) did not overlap the theoretical AD₅₀.^{27,28} NTB (1) + norBNI (25) was 6-fold more potent in the presence of the other antagonist, while the combination norBNI (1) + β -FNA (3) was 9 times more potent when compared to being tested individually. (Supporting Information Figure S3 (parts A and B)). These data are consistent with the cell-based targeting by 3 to both KOR-DOR and KOR-MOR putative heteromers that were observed in the calcium mobilization studies.

Compound 3 Does Not Produce Tolerance or Physical Dependence. Chronic tolerance was evaluated using a modified methodology of Fairbanks et al.²⁹ 3 was administered i.t. (ED_{80–90} dose) twice on day 1 and day 2. On the third day, the mice were injected with the same dose and retested to compare the ED_{80–90} value. Tolerance is indicated by a significantly higher ED₅₀ value. On day 3, compound 3 did not induce significant tolerance, as its ED₅₀ was 29.47 pmol (16.97–51.23) compared to the control ED₅₀ [22.05 pmol (14.71–33.05)] (Supporting Information Figure S4).

Evaluation of physical dependence was conducted in mice that were injected sc with 3, 3 times daily for 4 days (2.5–10 mg/kg) and 10 mg/kg on day 5, followed 3 h later by a single 10 or 50 mg/kg dose of sc naloxone.³⁰ The degree of physical dependence was estimated by counting the number of jumps over a 10 min period. When compared to a morphine control (71 jumps), 3 (14 jumps) failed to reveal significant physical dependence with either 10 or 50 mg/kg of naloxone (Supporting Information Figure S5).

Place Conditioning Studies of 3. Standard κ opioid agonists are known to be aversive in the dose range for antinociception.^{31–34} Because no drug-induced dependence of 3 was observed in the naloxone-induced jumping test, the effect of sc-administered 3 on place preference was measured in mice after four or eight days of conditioning. The most noteworthy feature in these experiments, was a robust dose-dependent place preference in the 0.3–10 mg/kg dose range (Figure 2A), which overlapped with the sc ED₅₀ dose for antinociception. A comparative study with the κ -selective ligand, salvinorin A,^{35–38} induced aversion at all doses tested (0.1, 0.3, and 1.0 mg/kg (Figure 2B)). As naloxone treatment of mice blocked the place preference induced by 3 (Figure 2C), it suggests that the rewarding effect of derivative 3 was mediated via opioid receptors (see also Supporting Information Table S3). This dose of naloxone (1.0 mg/kg) produced no aversion on its own.

Activation of Putative KOR-DOR Heteromers by 3 Does Not Lead to Recruitment of β -Arrestin2.

Compound 3 possessed the most favorable in vivo profile from the standpoint of parenteral and oral antinociception, lack of tolerance and dependence, and absence of aversion in conditioned place aversion. As adverse effects have been associated with β -arrestin2 recruitment,^{39–50} we investigated how 3 would affect such recruitment upon activation in the presence of singly expressing or coexpressing opioid receptors in HEK293 cells. Similar experiments also were carried out with standard μ , δ , and κ agonist ligands (DAMGO,⁵¹ DPDPE,⁵² U69593,⁵³ respectively) as controls. Cells were treated with immunofluorescent β -arrestin2 primary antibodies (goat) and secondary antigoat antibodies to image the receptors. A brighter cell surface membrane was the criterion for positive β -arrestin2 recruitment. Cells with singly expressing receptors showed recruitment when exposed to 3 or the three standard opioid ligands (Figure 3). However, with

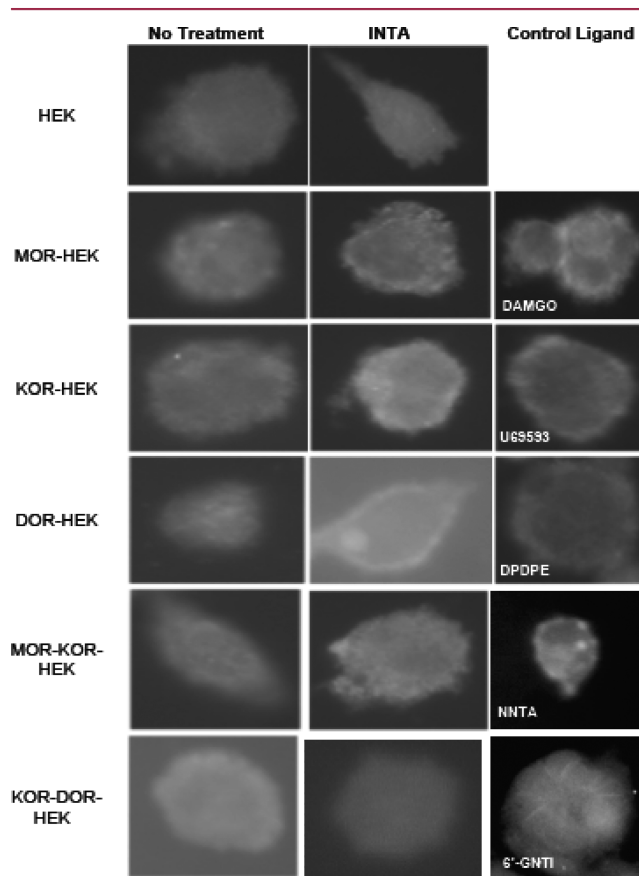


Figure 3. Representative high power fluorescent micrographs of control HEK293 cells and those singly and doubly expressing opioid receptors that were stained for β -arrestin2. INTA (3)- and NNTA-treated MOR-KOR HEK293 cells showed some recruitment of β -arrestin2, while INTA-treated KOR-DOR HEK293 cells showed no recruitment of β -arrestin2. 6'-GNTI-treated KOR-DOR HEK 293 cells exhibited little recruitment. Treatment of nontransfected HEK293 cells with 3, NNTA (1), 6'-GNTI (2), or any of the standard opioid control ligands did not induce β -arrestin2 recruitment (not shown).

KOR-DOR coexpressing cells, 3 did not recruit β -arrestin2 to cell membranes and MOR-KOR appeared to exhibit a reduced recruitment. Thus, the effect of 3 on β -arrestin2 clearly appears to be different for cells that coexpress KOR-DOR when compared to those expressing KOR-MOR, MOR, DOR, and KOR.

It has recently been reported that **2** inhibits recruitment of β -arrestin to KOR and possibly KOR-DOR in cultured cells.^{54–56} There are, however, notable selectivity differences as **3** inhibits recruitment only of KOR-DOR, leaving KOR unaffected. This difference can simply be explained by the involvement of different mechanisms for **3** and **2**, particularly because the ligands differ architecturally.

DISCUSSION

It is now generally recognized that following G protein activation, β -arrestin associates with the phosphorylated receptor and can function as a scaffold for the binding of a host of intracellular mediators that initiate other signaling pathways, thereby leading to a diverse signaling network.^{8–15} As this network has been considered to be a source of some adverse effects, it has been proposed that a viable approach to the development of drugs with fewer side effects can be accomplished by reducing, modifying, or preventing interaction of β -arrestin with the GPCR.^{39,40,42,43,45,46,56–58}

There is burgeoning evidence for the possible involvement of opioid receptor heteromers in promoting desired and/or adverse pharmacologic effects.^{16,59} For examples, early studies have suggested that interaction between μ and δ opioid receptors are obligatory for the development of tolerance and physical dependence.^{59a–c} More recent studies have provided additional support for this concept with bivalent ligands that target putative MOR-DOR heteromers.^{59d–h} Given evidence involving G protein switching from $G_{i/o}$ (MOR) to G_z (MOR-DOR) upon activation,⁶⁰ this could account for lack of side effects in some cases. Very recent studies with a μ agonist/mGluR5 antagonist bivalent ligand⁶¹ and with **1**,¹⁶ a ligand that selectively targets MOR-KOR heteromers are consistent with this concept. Thus, it occurred to us that the constitution of different opioid receptor heteromers could also affect recruitment of β -arrestin.^{62–66} On the basis of this concept, we have synthesized and screened ligands that selectively activate κ opioid receptors coexpressed with MOR and DOR in an effort to reduce or eliminate the well-known aversive effects associated with κ agonists. If some opioid receptor heteromers do not readily recruit β -arrestin upon activation, perhaps their adverse effects might be reduced or eliminated. In this regard, **1**, which selectively activates putative MOR-KOR heteromers, produces potent antinociception without tolerance or dependence. However, not all adverse effects were eliminated, as some aversion was noted at 10 times its ED_{50} dose.¹⁶ The study of **1** raised the possibility that its adverse effects might be due to a switch in G protein that differs from that involved in the activation of MOR-DOR, which in turn could reduce the recruitment of β -arrestin.

With the above concept in mind, we have synthesized and tested a focused library based on the structural requirements of the μ - κ agonist, **1**,¹⁶ and **2**,¹⁷ which was reported to activate KOR-DOR heteromer. After initial screening in HEK-293 cells singly expressing MOR-, KOR-, and DOR- or coexpressing MOR-DOR, KOR-MOR-, and KOR-DOR using the calcium mobilization assay, we have found that the β -naltrexamine derivative **3** is highly potent in activating both KOR-MOR and KOR-DOR heteromers in the pM range (Figure 1). In this regard, it is noteworthy that small structural changes of **3** produced substantial changes in potency or selectivity.

In mice, the i.t. potency of **3** was equivalent to that of morphine. However, by the icv route, **3** was ~60-fold less potent. Significantly, compound **3** produced neither tolerance nor dependence. Other members of the series were either less

potent and/or produced tolerance. In view of the favorable in vivo pharmacological profile of **3**, i.t. antagonism studies were performed using standard opioid antagonists. As the ED_{50} of **3** was strongly right-shifted by a single dose of naloxone, it appears that its action is mediated via opioid receptors. In an effort to evaluate the in vivo selectivity of **3** at opioid receptors, we investigated the effect of κ (norBNI), δ (NTB), and μ (β -FNA) antagonists on antinociception when administered separately or in combination. The observation that norBNI/NTB or norBNI/ β -FNA combinations exhibited synergism is consistent with interaction of **3** with KOR-DOR and KOR-MOR heteromers. These data support the selectivity profile of **3** shown in the calcium mobilization assay (Figure 1).

Immunofluorescent imaging of β -arrestin2 recruitment in HEK293 cells singly or coexpressing opioid receptors revealed that receptor activation by **3** led to recruitment of β -arrestin in all these cell lines except KOR-DOR (Figure 3). The observation that the KOR-DOR/KOR-MOR agonist (**3**) induced recruitment of β -arrestin in KOR-MOR cells but did not produce aversion in mice (Figure 2A) was intriguing because the κ - μ selective agonist, **1**, has been reported to be aversive at 10 \times its ED_{50} dose.¹⁶

The fact that **3** exhibited conditioned place preference that was reversed by naloxone suggested that the reversal was due to interaction of naloxone with a putative opioid receptor heteromer. However, in light of the absence of naloxone-induced jumping, the place preference does not appear to be coincidental with dependence. Because compound **3** targets both KOR-MOR and KOR-DOR in HEK293 cells, some aversion would be expected in the conditioned place preference assay if these putative heteromers behave independently. This raises the possibility that perhaps the KOR-MOR and KOR-DOR that are targeted by **3** do not behave independently but rather function as an higher-order oligomer, as there are reports for the existence of tetramers among class A GPCRs.^{2,67–69} Such a higher-order organization might lead to a pharmacologic profile that would differ from individual heterodimers that are not intimately associated. In any case, the present study suggests that targeting of KOR-DOR heteromer may be a viable approach to the design of analgesics devoid of the side effects associated with the presently employed analgesics. These data also reinvigorate the interest for the development of κ or κ -associated agonist therapeutics that are not peripherally restricted.

These and prior studies suggest that targeting putative κ -opioid receptor heteromers is a viable approach to the development of analgesics that are free of side effects through modulation or inhibition of β -arrestin2-dependent signaling pathways that may promote adverse effects. Also, the combinatorial possibilities that exists for KOR-containing heteromers affords more targets than homomers. In this regard, nearly two dozen heteromers containing opioid protomers or opioid and nonopioid protomers have been reported in cultured cells.^{1–6,61,70–77} From this perspective, the targeting of opioid heteromers has the potential of identifying new leads for development of ligands devoid of adverse effects for management of pain.

EXPERIMENTAL SECTION

Chemistry. For material and method, see ref 18.

Analytical data confirmed the purity of the products was $\geq 95\%$.

General Protocol for Amide Bond Formation. As reported previously,¹⁸ 6- β -naltrexamine (1 equiv) was dissolved in DCM and benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) (2 equiv), and the appropriate aryl carboxylic acid

(2.4 equiv) was added. *N,N*-Diisopropylethylamine (DIPEA) (2.8 equiv) was then added to the mixture. The solution was stirred for 2–16 h at room temperature and concentrated to dryness. The resulting solid was dissolved in anhydrous methanol (3 mL) and K_2CO_3 (7.3 equiv) was added. The mixture was stirred at room temperature for 1 h, concentrated, and purified by SiO_2 chromatography. The targeted compounds were then converted into hydrochloride salt for pharmacological evaluation.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(2'-indolyl)acetamido]morphinan (3, INTA). Purification by flash chromatography [DCM/MeOH 99/1] and then precipitation from an acetone/hexanes mixture provided 3 as a white solid. Yield 78%. 1H NMR (DMSO- d_6) δ : 0.11 (m, 2H), 0.46 (m, 2H), 0.84 (m, 1H), 1.25–1.60 (m, 4H), 1.82–1.99 (m, 2H), 2.17 (m, 1H), 2.33 (m, 2H), 2.58 (m, 2H), 3.02 (m, 2H), 3.69 (m, 1H), 4.68 (d, 1H, J = 7.8 Hz), 4.90 (bs, 1H, OH-14), 6.52–6.57 (m, 2H, H_{11} , H_2), 7.01 (m, 1H), 7.15 (m, 2H), 7.40 (d, 1H, J = 7.6 Hz, H), 7.61 (d, 1H, J = 8.0 Hz), 8.63 (d, 1H amide, J_{NH} = 8.3 Hz), 9.03 (bs, 1H, OH-3), 11.51 (s, 1H, NH indole). ^{13}C NMR (DMSO- d_6) δ : 3.52, 3.64, 9.20, 22.13, 24.80, 30.04, 30.26, 43.65, 47.01, 51.10, 58.36, 61.72, 69.56, 90.65, 102.20, 112.22, 116.98, 118.38, 119.59, 121.46, 123.15, 123.45, 127.04, 131.32, 131.75, 136.33, 140.43, 142.03, 160.63; mp 232–234 °C. Anal. Calcd for $C_{29}H_{31}N_3O_4$: C, 71.73; H, 6.43; N, 8.65. Found: C, 71.65; H, 6.39; N, 8.32. ESI-TOF MS calculated for $C_{31}H_{32}N_2O_4$, m/z 485.574; found, 486.123 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[1'-*N*-methyl-(2'-indolyl)acetamido]morphinan (4). Purification by flash chromatography [hexanes/AcOEt 25/75] and then precipitation from an acetone/hexanes mixture provided 4 as a white solid. Yield 91%. 1H NMR (DMSO- d_6) δ : 0.01 (m, 2H), 0.40 (m, 2H), 0.71 (m, 1H), 1.11 (m, 1H), 1.36 (m, 2H), 1.51 (m, 2H), 1.76 (m, 1H), 2.05 (m, 2H), 2.22 (m, 2H), 2.88 (d, 1H, J = 8.4 Hz), 2.98 (d, 1H, J = 5.7 Hz), 3.92 (s, 3H), 4.01 (m, 1H), 4.42 (d, 1H, J = 7.9 Hz), 6.39 (d, 1H, J = 8.2 Hz), 6.63 (d, 1H, J = 8.1 Hz), 6.81 (s, 1H), 7.01 (t, 1H, J = 7.1 Hz), 7.13–7.17 (m, 2H), 7.22 (d, 1H, J = 8.3 Hz), 7.31 (d, 1H amide, J_{NH} = 8.9 Hz), 7.48 (d, 1H, J = 7.9 Hz). ^{13}C NMR (DMSO- d_6) δ : 3.78, 3.99, 9.40, 22.61, 22.33, 29.08, 31.59, 36.81, 43.93, 47.26, 50.02, 59.36, 62.33, 70.15, 92.53, 104.12, 110.06, 117.70, 118.97, 120.30, 121.82, 123.89, 124.24, 126.08, 130.68, 132.15, 139.05, 139.92, 143.28, 162.35; mp 196–197 °C. Anal. Calcd for $C_{30}H_{33}N_3O_4$: C, 72.13; H, 6.66; N, 8.41. Found: C, 72.23; H, 6.64; N, 8.39. ESI-TOF MS calculated for $C_{30}H_{33}N_3O_4$, m/z 499.601; found m/z , 500.123 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(3'-indolyl)acetamido]morphinan (5). Purification by flash chromatography [DCM/MeOH 99/1] and then precipitation from an acetone/hexanes mixture provided 5 as a white solid. Yield 82%. 1H NMR (CD $_3$ OD) δ : 0.22 (m, 2H), 0.57 (m, 2H), 0.91 (m, 1H), 1.47–1.73 (m, 4H), 1.98 (m, 1H), 2.28 (m, 2H), 2.74 (m, 2H), 3.12 (d, 1H, J = 8.1 Hz), 3.16 (d, 1H, J = 5.8 Hz), 3.93 (m, 1H), 4.64 (d, 1H, J = 7.8 Hz), 6.60 (d, 1H, J = 8.2 Hz), 6.64 (d, 1H, J = 8.2 Hz), 7.11–7.40 (m, 2H), 7.42 (d, 1H, J = 7.6 Hz), 7.91 (s, 1H), 7.61 (dd, 1H, J = 0.9 Hz, J = 6.9 Hz). ^{13}C NMR (CD $_3$ OD) δ : 3.99, 4.12, 14.44, 23.74, 25.76, 30.49, 31.29, 35.76, 49.45, 52.43, 58.75, 60.92, 63.89, 71.73, 93.41, 111.78, 112.73, 119.00, 120.21, 121.89, 121.96, 123.48, 125.44, 127.26, 128.65, 128.94, 138.08, 142.43, 143.87, 168.34; mp 170–174 °C. Anal. Calcd for $C_{29}H_{30}N_3O_4$: C, 71.73; H, 6.43; N, 8.65. Found: C, 71.65; H, 6.39; N, 8.32. ESI-TOF MS calculated for $C_{31}H_{32}N_2O_4$, m/z 485.574; found 486.124, (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(5'-indolyl)acetamido]morphinan (6). Purification by flash chromatography [DCM/MeOH 99/1] and then precipitation from an acetone/hexanes mixture provided 6 as a white solid. Yield 75%. 1H NMR (CD $_3$ OD) δ : 0.19 (m, 2H), 0.57 (m, 2H), 0.89 (m, 1H), 1.47–1.73 (m, 2H), 1.82–1.99 (m, 2H), 1.96 (m, 1H), 2.29 (m, 2H), 2.46 (m, 2H), 2.73 (m, 2H), 3.12 (d, 1H, J = 8.3 Hz), 3.22 (d, 1H, J = 5.9 Hz), 3.97 (m, 1H), 4.68 (d, 1H, J = 7.8 Hz), 6.55 (dd, 1H, J = 0.7 Hz, J = 3.1 Hz), 6.60 (d, 1H, J = 8.2 Hz), 6.65 (d, 1H, J = 8.1 Hz), 7.31 (d, 1H, J = 3.2 Hz), 7.42 (d, 1H, J = 8.5 Hz), 7.63 (dd, 1H, J = 1.7 Hz, J = 8.6 Hz), 8.14 (d, 1H, J = 1.2 Hz). ^{13}C NMR (CDCl $_3$) δ : 4.12, 4.19, 12.31, 23.62, 25.65, 31.31, 34.29, 48.34, 49.61, 53.21, 60.15, 63.82, 71.85, 93.26, 103.56, 111.95, 118.75, 120.07, 120.42, 121.44, 121.63 (2 \times), 122.57, 126.41, 127.21, 129.02, 135.61, 139.56, 143.82, 171.56; mp 170–172 °C. Anal. Calcd for $C_{29}H_{31}N_3O_4$: C, 71.73; H, 6.43; N, 8.65. Found: C, 72.65; H,

6.19; N, 8.12. ESI-TOF MS calculated for $C_{31}H_{32}N_2O_4$, m/z 485.574; found, 486.157 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(5'-chloro-2'-indolyl)acetamido]morphinan (7). Purification by flash chromatography [Hexanes/AcOEt 15/85] and then precipitation from an acetone/hexanes mixture provided 7 as a white solid. Yield 75%. 1H NMR (CDCl $_3$) δ : 0.14 (m, 2H), 0.55 (m, 2H), 0.86 (m, 1H), 1.08 (m, 2H), 1.36 (m, 2H), 1.82–1.99 (m, 2H), 2.17 (m, 1H), 1.94 (m, 2H), 2.39 (m, 2H), 3.02 (d, 1H, J = 8.3 Hz), 3.14 (d, 1H, J = 5.9 Hz), 3.47 (m, 1H), 4.55 (d, 1H, J = 7.8 Hz), 6.44 (s, 1H), 6.58 (d, 1H, J = 8.2 Hz), 6.74 (d, 1H, J = 8.1 Hz), 6.97 (d, 1H amide, J = 9.3 Hz), 7.20 (dd, 1H, J = 2.0 Hz, J = 8.7 Hz), 7.27 (d, 1H, J = 8.8 Hz), 7.49 (s, 1H), 9.92 (d, 1H, NH indole). ^{13}C NMR (CDCl $_3$) δ : 3.80, 4.01, 9.41, 24.93, 25.61, 33.94, 36.49, 47.46, 49.20, 50.27, 59.30, 62.24, 70.18, 93.21, 101.62, 112.87, 118.11, 119.61, 121.13, 124.53, 124.89, 126.08, 128.58, 130.63, 131.83, 134.53, 139.81, 142.61, 160.75; mp 178–180 °C. Anal. Calcd for $C_{29}H_{30}ClN_3O_4$: C, 66.98; H, 5.81; N, 8.08. Found: C, 66.72; H, 5.84; N, 7.89. ESI-TOF MS calculated for $C_{29}H_{30}ClN_3O_4$, m/z 518.182; found, 520.096 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(5'-fluoro-2'-indolyl)acetamido]morphinan (8). Purification by flash chromatography [hexanes/AcOEt 25/75] and then precipitation from an acetone/hexanes mixture provided 8 as a white solid. Yield 89%. 1H NMR (CD $_3$ OD) δ : 0.17 (m, 2H), 0.55 (m, 2H), 0.89 (m, 1H), 1.54–1.72 (m, 4H), 1.99 (m, 1H), 2.17–2.30 (m, 2H), 2.42 (m, 2H), 2.69 (m, 2H), 3.07 (d, 1H, J = 8.2 Hz), 3.14 (d, 1H, J = 5.9 Hz), 3.93 (m, 1H), 4.64 (d, 1H, J = 7.8 Hz), 6.23 (d, 1H, J = 8.2 Hz), 6.28 (d, 1H, J = 8.1 Hz), 6.63 (td, 1H, J = 2.5 Hz, J = 9.2 Hz), 6.73 (s, 1H), 6.92 (dd, 1H, J = 2.5 Hz, J = 9.6 Hz), 7.05 (m, 1H). ^{13}C NMR (CDCl $_3$) δ : 4.17, 4.51, 10.21, 23.56, 25.76, 31.33, 31.88, 45.37, 49.21, 52.94, 60.23, 63.75, 71.79, 93.12, 104.21, 106.56, 106.80, 114.09, 114.19, 118.66, 120.09, 125.43, 129.19, 132.53, 134.07, 134.92, 141.84, 143.72, 142.61, 163.54. ^{19}F NMR (CD $_3$ OD) δ –125.99; mp 178–180 °C. Anal. Calcd for $C_{29}FH_{30}N_3O_4$: C, 69.17; H, 6.00; N, 8.34. Found C, 69.59; H, 5.97; N, 8.45. ESI-TOF MS calculated for $C_{29}FH_{30}N_3O_4$, m/z 503.565; found, 504.036 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(2'-benzothiophene)acetamido]morphinan (9). Purification by flash chromatography [hexanes/AcOEt 25/75] and then precipitation from an acetone/hexanes mixture provided 9 as a white solid. Yield 77%. 1H NMR (CD $_3$ OD) δ : 0.17 (m, 2H), 0.53 (m, 2H), 0.87 (m, 1H), 1.25–1.62 (m, 4H), 1.99 (m, 1H), 2.12–2.29 (m, 2H), 2.47 (m, 2H), 2.73 (m, 2H), 3.06 (d, 1H, J = 8.3 Hz), 3.12 (d, 1H, J = 5.9 Hz), 3.89 (m, 1H), 4.63 (d, 1H, J = 7.8 Hz), 6.57 (d, 1H, J = 8.2 Hz), 6.69 (d, 1H, J = 8.2 Hz), 7.28–7.42 (m, 2H), 7.86–7.89 (m, 2H), 7.96 (s, 1H). ^{13}C NMR (CD $_3$ OD) δ : 4.13, 4.54, 11.62, 23.61, 25.87, 31.47, 32.78, 49.61, 50.83, 51.78, 53.93, 61.18, 64.74, 72.32, 93.83, 113.15, 114.52, 118.52, 120.89, 121.12, 122.94, 123.51, 123.64, 124.21, 124.83, 128.67, 134.57, 139.95, 143.80, 171.51; mp 192–194 °C. Anal. Calcd for $C_{29}H_{30}N_3O_4S$: C, 69.30; H, 6.02; N, 5.57. Found: C, 68.67; H, 5.74; N, 5.88. ESI-TOF MS calculated for $C_{29}H_{30}N_2O_4S$, m/z , 502.627; found, 503.869 (MH) $^+$.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(2'-benzofuryl)acetamido]morphinan (10). Purification by flash chromatography [hexanes/AcOEt 25/75] and then recrystallized from MeOH provided 10 as a white solid. Yield 92%. 1H NMR (CD $_3$ OD) δ : 0.19 (m, 2H), 0.56 (m, 2H), 0.93 (m, 1H), 1.44–1.70 (m, 4H), 2.04 (m, 1H), 2.19–2.32 (m, 2H), 2.45 (m, 2H), 2.71 (m, 2H), 3.09 (d, 1H, J = 8.3 Hz), 3.16 (d, 1H, J = 5.9 Hz), 3.96 (m, 1H), 4.69 (d, 1H, J = 7.8 Hz), 6.61 (d, 1H, J = 8.2 Hz), 6.66 (d, 1H, J = 8.1 Hz), 7.32 (t, 1H, J = 7.6 Hz), 7.46–7.49 (m, 2H), 7.62 (d, 1H, J = 8.2 Hz), 7.73 (d, 1H, J = 7.7 Hz). ^{13}C NMR (CD $_3$ OD) δ : 4.12, 4.57, 11.71, 23.59, 25.56, 31.36, 31.61, 48.78, 49.82, 51.48, 52.88, 60.16, 63.76, 71.76, 92.82, 111.38, 112.84, 118.68, 119.87, 119.99, 120.15, 121.47, 122.78, 123.74, 124.89, 128.19, 133.47, 139.74, 142.81, 168.42; mp 162–164 °C. Anal. Calcd for $C_{29}H_{30}N_2O_5$: C, 71.59; H, 6.21; N, 5.76. Found: C, 71.47; H, 6.15; N, 5.71. ESI-TOF MS calculated for $C_{29}H_{30}N_2O_5$, m/z , 486.564; found, 487.04 (MH) $^+$.

Intracellular Calcium Release. The assay was performed as described previously.¹⁸ Briefly, HEK-293 cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium supplemented with 10% bovine calf serum and Pen/Strep antibiotics. These cells were transiently

transfected with 200 ng/20000 cells of the opioid receptor cDNA using OptiMEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol (1:2 wt/vol ratio for DNA:Lipofectamine; 16 μ g DNA, 32 μ L Lipofectamine for singly expressed receptors, 12 μ g of each receptor DNA (24 μ g total), 48 μ L Lipofectamine for doubly expressed receptors). The cells were seeded into 96-well plates (half area; Corning) at 20000 cells/well after 24 h and assayed 48 h after transfection using the FLIPR calcium kit (Molecular devices) in a Flexstation-III apparatus (Molecular devices).

Animals. Male ICR-CD1 mice (17–25 g; Harlan, Madison, WI) employed in the testing were housed in groups of eight in a temperature- and humidity-controlled environment with unlimited access to food and water. They were maintained on a 12 h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Minneapolis, MN).

Antinociceptive Testing. For the measurement of antinociception, the tail-flick latency assay^{19,20} was employed (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio). The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat was set so that the mouse flicks its tail within 2–3 s. The test latency was measured 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 as the percent maximal possible effect (%MPE): %MPE = (test – control/10 – control) \times 100²¹. Three groups of 8–10 mice were used for each dose–response curve, and each mouse was used only once. Peak times are established by measuring the antinociception 5, 10, 20, 30, and 60 min after administration of the compound being tested. ED₅₀ values with 95% confidence intervals (CI) were computed with GraphPad Prism 4 by using nonlinear regression methods.

Antagonist Synergy. The AD₅₀ for each antagonist was measured by changing the dose of the antagonist and keeping the agonist dose (ED₉₀) constant.^{27,28} The theoretical AD₅₀ was based on the ratio of the two antagonists being tested together. The theoretical was then compared to the actual AD₅₀ measured if there was no overlapping within the confident limits the compound was said to be synergistic.

Conditioned Place Preference. Place conditioning procedures are used to assess the rewarding and aversive effects of psychoactive drugs.³⁴ An unconditioned stimulus (US; drug) was paired with a conditioned stimulus (CS) and rewarding or aversive effects of the US cause a preference for or against the CS (context) to develop. Similar protocols have recently been used in rats to demonstrate both rewarding³⁶ and aversive properties^{37,38} of the κ opioid receptor-selective agonist salvinorin A.

Apparatus. A two-chambered place preference apparatus (22 \times 45 \times 20 cm³) was used. The test chamber was divided into two compartments separated by an opaque wall with a guillotine-style door. The floors of the two experimental chambers differed in the floor construction. One floor was made of a series of 2.3 mm stainless steel rods, centered every 6.4 mm, and was termed the “rod” floor. The other floor, called the “mesh” floor, was made up of thin wire mesh with openings of 2.5 mm \times 2.5 mm. This apparatus allowed for an unbiased (rod vs mesh) experimental design. All data was collected by video camera, and movements were analyzed by ANY-maze (Stoelting Co., Wood Dale, IL).

Preconditioning. Prior to the beginning of the experiment, mice were habituated to the experimenter through 3 days of handling and saline injections. After habituation to handling and injections, initial chamber bias was determined for each mouse by placing them in the test chamber and allowed free exploration of the apparatus for 20 min in a “pre-test”. Total time spent in rod- and mesh-floored sides was recorded and analyzed.

Conditioning. Three experiments were conducted: (1) INTA dose–response, (2) salvinorin A dose–response, and (3) antagonism of INTA with naloxone. In experiment 1, mice were assigned to have INTA (0.3, 1.0, 3.0, or 10.0 mg/kg, sc), vehicle (saline w/1% DMSO) associated with either the rod- or mesh-floored chamber (CS+), and saline paired to the other chamber (CS-chamber). In experiment 2, mice were assigned to have salvinorin A (0.3, 1.0, or 3.0 mg/kg, sc) or vehicle associated with either the rod- or mesh-floored chamber (CS+ chamber)

and saline paired to the other chamber (CS-chamber). In experiment 3, mice were given an injection of naloxone (1.0 sc) or vehicle and a second injection of INTA (3.0 mg/kg) or vehicle prior to CS+ sessions and saline prior to CS-sessions. All drug group assignments were done pseudorandomly and counterbalanced for pretest performance. In the conditioning trials, each subject was injected with the drug or saline 20 min prior to being confined to the corresponding paired chamber for 30 min. A total of four drug and four saline trials were performed with one conditioning trial performed per day alternating between drug and saline (8 total days).

Test Sessions. Two tests were performed. On the day following the fourth conditioning session, mice were tested as before in the preconditioning test (free exploration for 20 min). A second test was to be performed after the eighth conditioning session. Place preference induced by the US was determined by the difference in time spent in the CS+ chamber during the test session as compared to pretest. Experiments 1 and 2 were analyzed across test sessions, and two-way ANOVA (with Bonferroni-corrected *t* test comparisons) was used to determine the main effects of conditioning and drug (INTA or salvinorin A) and the interaction of the two. Because of the multiple drug treatments in experiment 3, test 2 was separately analyzed with two-way ANOVA for the main effects of naloxone and INTA treatments and the interaction of the two.

Immunofluorescence Study for β -arrestin2 Recruitment. HEK293 cells (100 mm² tissue culture plate, 80–100% confluent) were transiently transfected with MOP, KOP, or DOP (16 μ g DNA in 32 μ L Lipofectamine 2000 (Invitrogen)), or MOP + KOP, MOP + DOP, or KOP + DOP (12 μ g DNA for each receptor in 48 μ L of Lipofectamine 2000). After 24 h, cells were diluted 1:100 and replated in 8-well tissue culture slides (B-D Falcon) and incubated overnight. Ligands were diluted initially in 10% DMSO and 90% water. Serial dilutions were prepared in Dulbecco's medium without serum and added to the wells to achieve the concentrations listed. Ligands were incubated with the cells for 60 min at 37 °C. The media was then gently removed, and cells were fixed using 4% paraformaldehyde in 0.2 M Sorenson's buffer for 20 min. The fixative was gently removed, and the cells were washed three times with 1 \times phosphate buffered saline (PBS). Primary and secondary antibodies were diluted in 0.3% Triton X-100, 1% normal donkey serum, 1% bovine serum albumin, and 0.01% sodium azide. Anti- β -arrestin2 (goat) antibodies (R&D Systems) were added to each well at a concentration of 1 μ g/mL and incubated at 4 °C overnight on an oscillating shaker plate. Cells were washed as above and incubated with antigoat Northern Lights 493 (R&D Systems) for 30 min at room temperature. The cells were washed as above, except that during the last wash, DAPI (1 μ g/mL) was added and incubated for 15 min at room temperature. The cells were mounted using i-Brite Plus (Neuromics) mounting medium and covered with a glass coverslip. High power images were recorded using a Leica DMI4000 fluorescent microscope and processed using ImageJ software (NIH).

■ ASSOCIATED CONTENT

● Supporting Information

EC₅₀s for the calcium mobilization assay, competitive binding for 3, and antagonist, tolerance, and dependence study data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GPCR, G-protein-coupled receptor; KOR, κ -opioid receptor; MOR, μ -opioid receptor; DOR, δ -opioid receptor; INTA, *N*-2'-indolyl- β -naltrexamine; NNNTA, *N*-2'-naphthoyl- β -naltrexamine; 6'-GNTI, 6'-guanidinonaltrexamine; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; HEK293, human embryonic kidney 293; FLIPR, fluorescence imaging plate reader; RFU, relative fluorescence units; icv, intracerebroventricular; i.t., intrathecal; MPE, percentage of maximum effect; DAMGO, [D-Ala², N-MePhe⁴, Gly⁵]enkephalin; β -FNA, β -funaltrexamine; NTB, naltriben; DPDPE, [D-Pen², D-Pen⁵]enkephalin; NorBNI, norbinaltorphimine; AD, antagonist dose; sc, subcutaneous; CPP, condition place preference

■ REFERENCES

- (1) (a) Milligan, G. G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol. Pharmacol.* **2004**, *66*, 1–7. (b) Rachid, A. J.; O'Dowd, B. F.; George, S. R. Diversity and complexity of signaling through peptidergic G protein-coupled receptors. *Endocrinology* **2005**, *145*, 2645–2652.
- (2) Hiller, C.; Kuehnhorn, J.; Gmeiner, P. Class A G protein-coupled receptor (GPCR) dimers and bivalent ligands. *J. Med. Chem.* **2013**, *56* (17), 6542–6559.
- (3) Ferre, S.; Baler, R.; Bouvier, M.; Caron, M. G.; Devi, L. A.; Durroux, T.; Fuxe, K.; George, S. R.; Javitch, J. A.; Lohse, M. J.; Mackie, K.; Milligan, G.; Pfeiffer, K. D. G.; Pin, J.-P.; Volkow, N.; Waldhoer, M.; Woods, A. S.; Franco, R. Building a new conceptual framework for receptor heteromers. *Nature Chem. Biol.* **2009**, *5*, 131–134.
- (4) Angers, S.; Salahpour, A.; Bouvier, M. Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 409–435.
- (5) Gomes, I.; Jordan, B. A.; Gupta, A.; Rios, C.; Trapaidze, N.; Devi, L. A. G protein-coupled receptor dimerization: implications in modulating receptor function. *J. Mol. Med.* **2001**, *79*, 226–242.
- (6) (a) Jordan, B. A.; Devi, L. A. G protein-coupled receptor heterodimerization modulates receptor function. *Nature* **1999**, *399*, 697–700. (b) Gomes, I.; Jordan, B. A.; Gupta, A.; Trapaidze, N.; Nagy, V.; Devi, L. A. Heterodimerization of μ and δ opioid receptors: a role in opiate synergy. *J. Neurosci.* **2000**, *20* (22), RC110/1–RC110/5. (c) Gomes, I.; Gupta, A.; Filipovska, J.; Szeto, H. H.; Pintar, J. E.; Devi, L. A. A role for heterodimerization of μ and δ opiate receptors in enhancing morphine analgesia. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 5135–5139. (d) Chakrabarti, S.; Liu, N.-J.; Gintzler, A. R. Formation of opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (46), 20115–20119. (e) Ong, E. W.; Cahill, C. M. Molecular perspectives for μ / δ opioid receptor heteromers as distinct, functional receptors. *Cells* **2014**, *3* (1), 152–179. (f) Constantino, C. M.; Gomes, I.; Stockton, S. D.; Lim, M. P.; Devi, L. A. Opioid receptor heteromers in analgesia. *Expert Rev. Mol. Med.* **2012**, *14*, e9. (g) George, S. R.; Fan, T.; Xie, Z.; Tse, R.; Tam, V.; Varghese, G.; O'Dowd, B. F. Oligomerization of μ - and δ -opioid receptors. Generation of novel functional properties. *J. Biol. Chem.* **2000**, *275*, 26128–26135.
- (7) (a) George, S. R.; Fan, T.; Xie, Z.; Tse, R.; Tam, V.; Varghese, G.; O'Dowd, B. F. Oligomerization of μ - and δ -opioid receptors. Generation of novel functional properties. *J. Biol. Chem.* **2000**, *275*, 26128–26135. (b) Gomes, I.; Filipovska, J.; Jordan, B. A.; Devi, L. A. Oligomerization of opioid receptors. *Methods* **2002**, *27*, 358–365. (c) Wang, D.; Sun, X.; Bohn, L. M.; Sade, W. Opioid receptor homo- and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer. *Mol. Pharmacol.* **2005**, *67*, 2173–2184.
- (8) Khelashvili, G.; Dorff, K.; Shan, J.; Camacho-Artacho, M.; Skrabanek, L.; Vroiling, B.; Bouvier, M.; Devi, L. A.; George, S. R.; Javitch, J. A.; Lohse, M. J.; Milligan, G.; Neubig, R. R.; Palczewski, K.; Parmentier, M.; Pin, J.-P.; Vriend, G.; Campagne, F.; Filizola, M. GPCR-OKB: the G protein-coupled receptor oligomer knowledge base. *Bioinformatics* **2010**, *26*, 1804–1805.
- (9) Ritter, S. L.; Hall, R. A. Fine-tuning of GPCR activity by receptor-interacting proteins. *Nature Rev. Mol. Cell Biol.* **2009**, *10*, 819–830.
- (10) Hoffmann, C.; Zörn, A.; Bünnemann, M.; Lohse, M. J. Conformational changes in G protein-coupled receptors: the quest for functionally selective conformations is open. *Br. J. Pharmacol.* **2008**, *153*, S358–S366.
- (11) (a) Kenakin, T. P. Biased signalling and allosteric machines: new vistas and challenges for drug discovery. *Br. J. Pharmacol.* **2012**, *165*, 1659–1669. (b) Kenakin, T.; Christopoulos, A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nature Rev. Drug Discovery* **2013**, *12* (3), 205–216.
- (12) (a) Bohn, L. M. *Functional Selectivity of G Protein-Coupled Receptor Ligands*, 1st ed.; Neve, K. A., Ed.; Humana Press Inc.: Totowa, NJ, 2009; pp 71–85. (b) Reiter, E.; Ahn, S.; Shukla, A. K.; Lefkowitz, R. J. Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annu. Rev. Pharmacol. Toxicol.* **2012**, *52*, 179–197.
- (13) Pradhan, A. A.; Smith, M. L.; Kieffer, B. L.; Evans, C. J. Ligand-directed signalling within the opioid receptor family. *Br. J. Pharmacol.* **2012**, *167*, 960–969.
- (14) Rozenfeld, R.; Devi, L. A. Exploring the role for heteromerization in GPCR signalling specificity. *Biochem. J.* **2011**, *433*, 11–18.
- (15) Whalen, E. J.; Rajagopal, S.; Lefkowitz, R. J. Therapeutic potential of β -arrestin- and G protein-biased agonists. *Trends Mol. Med.* **2011**, *17*, 126–139.
- (16) Yekkirala, A. S.; Lunzer, M. M.; McCurdy, C. R.; Powers, M. D.; Kalyuzhny, A. E.; Roerig, S. C.; Portoghese, P. S. *N*-Naphthoyl-beta-naltrexamine (NNNTA), a highly selective and potent activator of μ / κ -opioid heteromers. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (12), 5098–5103.
- (17) Waldhoer, M.; Fong, J.; Jones, R. M.; Lunzer, M. M.; Sharma, S. K.; Kostenis, E.; Portoghese, P. S.; Whistler, J. L. A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 9050–9055.
- (18) Le Naour, M.; Lunzer, M. M.; Powers, M. D.; Portoghese, P. S. Opioid activity of spinally selective analogues of *N*-naphthoyl- β -naltrexamine in HEK-293 cells and mice. *J. Med. Chem.* **2012**, *55* (2), 670–677.
- (19) D'Amour, F. E.; Smith, D. L. A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.* **1941**, *72*, 74–79.
- (20) Dewey, W. L.; Harris, L. S.; Howes, J. F.; Nuite, J. A. 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.* **1970**, *175*, 435–442.
- (21) Harris, L. S.; Pierson, A. K. Narcotic antagonists in the benzomorphan series. *J. Pharmacol. Exp. Ther.* **1964**, *143*, 141–148.
- (22) Portoghese, P. S.; Lipkowski, A. W.; Takemori, A. E. Binaltorphimine and nor-binaltorphimine, potent and selective kappa-opioid receptor antagonists. *Life Sci.* **1987**, *40*, 1287–1292.
- (23) Takemori, A. E.; Ho, B. Y.; Naeseth, J. S.; Portoghese, P. S. *nor*-Binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 255–258.
- (24) Portoghese, P. S.; Nagase, H.; MaloneyHuss, K. E.; Lin, C. E.; Takemori, A. E. Role of spacer and address components in peptidomimetic delta opioid receptor antagonists related to naltrindole. *J. Med. Chem.* **1991**, *34*, 1715–1720.
- (25) Ward, S. J.; Portoghese, P. S.; Takemori, A. E. Pharmacological profiles of beta-funaltrexamine (beta-FNA) and beta-chlornaltrexamine (beta-CNA) on the mouse vas deferens preparation. *Eur. J. Pharmacol.* **1982**, *80*, 377–384.
- (26) Ward, S. J.; Portoghese, P. S.; Takemori, A. E. Pharmacological characterization in vivo of the novel opiate, beta-funaltrexamine. *J. Pharmacol. Exp. Ther.* **1982**, *220*, 494–498.

- (27) Guo, X.-H.; Fairbanks, C. A.; Stone, L. S.; Loh, H. H. DPDPE-UK14,304 synergy is retained in mu-opioid receptor knockout mice. *Pain* **2003**, *104*, 209–217.
- (28) Tang, Y.; Yang, J.; Lunzer, M. M.; Powers, M. D.; Portoghese, P. S. A κ opioid pharmacophore becomes a spinally selective κ - δ agonist when modified with a basic extender arm. *ACS Med. Chem. Lett.* **2011**, *2* (1), 7–10.
- (29) Fairbanks, C. A.; Kitto, K. F.; Nguyen, H. O.; Stone, L. S.; Wilcox, G. L. Clonidine and dexmedetomidine produce antinociceptive synergy in mouse spinal cord. *Anesthesiology* **2009**, *110* (3), 638–647.
- (30) Ayman, O. S.; Sharif, El-K.; Sharif, S. I. The influence of various experimental conditions on the expression of naloxone-induced withdrawal symptoms in mice. *Gen. Pharm.* **2004**, *25* (7), 1505–1510.
- (31) Suzuki, T. Conditioned place preference in mice. *Methods Find. Exp. Clin. Pharmacol.* **1996**, *18*, 75–83.
- (32) Wadenberg, M. L. A review of the properties of spiradoline: a potent and selective kappa-opioid receptor agonist. *CNS Drug Rev.* **2003**, *9*, 187–198.
- (33) Funada, M.; Suzuki, T.; Narita, M.; Misawa, M.; Nagase, H. Blockage of morphine reward through the activation of κ -opioid receptors in mice. *Neuropharmacology* **1993**, *32*, 1315–1323.
- (34) Cunningham, C. L.; Gremel, C. M.; Groblewski, P. A. Drug-induced conditioned place preference and aversion in mice. *Nature Protoc.* **2006**, *1* (4), 1662–1670.
- (35) (a) Shippenberg, T. S.; Zapata, A.; Chefer, V. I. Dynorphin and the pathophysiology of drug addiction. *Pharmacol. Ther.* **2007**, *116*, 306–321; (b) Bruchas, M. R.; Land, B. B.; Chavkin, C. The dynorphin/kappa-opioid receptor impacts the pathophysiology and behavior of substance use. *Brain Res.* **2010**, *16*, 44–55.
- (36) Braidia, D.; Limonta, V.; Capurro, V.; Fadda, P.; Rubino, T.; Mascia, P.; Zani, A.; Gori, E.; Fratta, W.; Parolaro, D.; Sala, M. Involvement of kappa-opioid and endocannabinoid system on salvinorin A-induced reward. *Biol. Psychiatry* **2008**, *63* (3), 286–292.
- (37) Carlezon, W. A., Jr.; Beguin, C.; DiNieri, J. A.; Baumann, M. H.; Richards, M. R.; Todtenkopf, M. S.; Rothman, R. B.; Ma, Z.; Lee, D. Y.-W.; Cohen, B. M. Depressive-like effects of the kappa-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. *J. Pharmacol. Exp. Ther.* **2006**, *316* (1), 440–447.
- (38) Zhang, Y.; Butelman, E. R.; Schlussman, S. D.; Ho, A.; Kreek, M. J. Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. *Psychopharmacology (Berlin)* **2005**, *179* (3), 551–558.
- (39) Yang, C.-H.; Huang, W.-W.; Chen, K.-H.; Chen, Y.-S.; Sheen-Chen, S.-M.; Lin, C.-R. Antinociceptive potentiation and attenuation of tolerance by intrathecal β -arrestin2 small interfering RNA in rats. *Br. J. Anaesth.* **2011**, *107*, 774–781.
- (40) Bohn, L. M.; Lefkowitz, R. J.; Gainetdinov, R. R.; Peppel, K.; Caron, M. G.; Lin, F. T. Enhanced morphine analgesia in mice lacking beta-arrestin2. *Science* **1999**, *286*, 2495–2498.
- (41) Bohn, L. M.; Gainetdinov, R. R.; Lin, F. T.; Lefkowitz, R. J.; Caron, M. G. Mu-opioid receptor desensitization by beta-arrestin2 determines morphine tolerance but not dependence. *Nature* **2000**, *408*, 720–723.
- (42) Bohn, L. M.; Lefkowitz, R. J.; Caron, M. G. Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin2 knock-out mice. *J. Neurosci.* **2002**, *22*, 10494–10500.
- (43) Raehal, K. M.; Walker, J. K.; Bohn, L. M. Morphine side-effects in beta-arrestin2 knock-out mice. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1195–1201.
- (44) Raehal, K. M.; Bohn, L. M. The role of beta-arrestin 2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* **2011**, *60*, 58–65.
- (45) Groer, C. E.; Schmid, C. L.; Jaeger, A. M.; Bohn, L. M. Agonist-directed interactions with specific beta-arrestins determine mu-opioid receptor trafficking, ubiquitination, and dephosphorylation. *J. Biol. Chem.* **2011**, *286* (36), 31731–31741.
- (46) Hales, T. G. Arresting the development of morphine tolerance and dependence. *Br. J. Anaesth.* **2011**, *107* (5), 653–655.
- (47) Raehal, K. M.; Schmid, C. L.; Groer, C. E.; Bohn, L. M. Functional selectivity at the μ -opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol. Rev.* **2011**, *63* (4), 1001–1019.
- (48) Raehal, K. M.; Bohn, L. M. The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* **2011**, *60* (1), 58–65.
- (49) Zuo, Z. The role of opioid receptor internalization and β -arrestins in the development of opioid tolerance. *Anesth. Analg.* **2005**, *101*, 728–734.
- (50) Bruchas, M. R.; Chavkin, C. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology (Berlin)* **2010**, *210* (2), 137–147.
- (51) Handa, B. K.; Land, A. C.; Lord, J. A.; Morgan, B. A.; Rance, M. J.; Smith, C. F. Analogues of beta-LPH61–64 possessing selective agonist activity at mu-opiate receptors. *Eur. J. Pharmacol.* **1981**, *70*, 531–540.
- (52) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine enkephalins possess highly improved specificity toward delta opioid receptors. *Proc. Natl. Acad. Sci. U. S. A.* **1983**, *80*, 5871–5874.
- (53) Lahti, R. A.; Mickelson, M. M.; McCall, J. M.; Von Voigtlander, P. F. 3H]U-69593 a highly selective ligand for the opioid kappa receptor. *Eur. J. Pharmacol.* **1985**, *109*, 281–284.
- (54) Rives, M.-L.; Rossillo, M.; Liu-Chen, L.-Y.; Javitch, J. A. 6'-Guanidinonaltrindole (6'-GNTI) is a G protein-coupled biased κ -opioid receptor agonist that inhibits arrestin recruitment. *J. Biol. Chem.* **2012**, *287* (32), 27050–27054.
- (55) Schmid, C. L.; Streicher, J. M.; Groer, C. E.; Munro, T. A.; Zhou, L.; Bohn, L. Functional selectivity of 6'-guanidinonaltrindole (6'-GNTI) at κ -opioid receptors in striatal neurons. *J. Biol. Chem.* **2013**, *288* (31), 22387–22398.
- (56) Groer, C. E.; Tidgewell, K.; Moyer, R. A.; Harding, W. W.; Rothman, R. B.; Prisinzano, T. E.; Bohn, L. M. An opioid agonist that does not induce m-opioid receptor–arrestin interactions or receptor internalization. *Mol. Pharmacol.* **2007**, *71* (2), 549–557.
- (57) DeWire, S. M.; Yamashita, D. S.; Rominger, D. H.; Liu, G.; Cowan, C. L.; Graczyk, T. M.; Chen, X. T.; Pitis, P. M.; Gotchev, D.; Yuan, C.; Koblish, M.; Lark, M. W.; Violin, J. D. A G protein-biased ligand at the μ -opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J. Pharmacol. Exp. Ther.* **2013**, *344* (3), 708–717.
- (58) Zhou, L.; Lovell, K. M.; Frankowski, K. J.; Slauson, S. R.; Phillips, A. M.; Streicher, J. M.; Stahl, E.; Schmid, C. L.; Hodder, P.; Madoux, F.; Cameron, M. D.; Prisinzano, T. E.; Aube, J.; Bohn, L. M. Development of functionally selective, small molecule agonists at kappa opioid receptors. *J. Biol. Chem.* **2013**, *288* (51), 36703–36716.
- (59) (a) Abdel-Hamid, E. E.; Sultana, M.; Portoghese, P. S.; Takemori, A. E. Selective blockage of delta opioid receptors prevents the development of morphine tolerance in mice. *J. Pharm. Exp. Ther.* **1991**, *258*, 299–303. (b) Kest, B.; Lee, C. E.; McLennore, G. L.; Inturrisi, C. E. An antisense oligodeoxynucleotide to the delta opioid receptor (DOR-1) inhibits morphine tolerance and acute dependence in mice. *Brain Res. Bull.* **1996**, *39*, 185–189. (c) Zhu, Y.; King, M. A.; Schuller, A. G.; Nitsche, J. F.; Reidl, M.; Elde, R. P.; Unterwald, E.; Pasternak, G. W.; Pintar, J. E. Retention of supraspinal morphine-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* **1999**, *24*, 243–252. (d) Daniels, D. J.; Lenard, N. R.; Etienne, C. L.; Law, P.-Y.; Roerig, S. C.; Portoghese, P. S. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 19208–19213. (e) Lenard, N. R.; Daniels, D. J.; Portoghese, P. S.; Roerig, S. C. Absence of conditioned place preference or reinstatement with bivalent ligands containing mu-opioid receptor agonist and delta-opioid receptor antagonist. *Eur. J. Pharmacol.* **2007**, *566*, 75–82. (f) Yekkirala, A. S.; Kalyuzhny, A. E.; Portoghese, P. S. Standard opioid agonists activate heteromeric opioid receptors:

- evidence for morphine and [D-Ala²,MePhe⁴Glyol⁵]enkephalin as selective μ - δ agonists. *ACS Chem. Neurosci.* **2010**, *1*, 146–154.
- (g) Aceto, M. D.; Harris, L. S.; Negus, S. S.; Banks, M. L.; Hughes, L. D.; E. Akgun, E.; Portoghese, P. S. MDAN-21: a bivalent opioid ligand containing mu-agonist and delta-antagonist pharmacophores and its effects in rhesus monkeys. *Int. J. Med. Chem.* **2012**, *30*, 4617–4624.
- (h) Yekkirala, A. S.; Banks, M. L.; Lunzer, M. M.; Negus, S. S.; Rice, K. C.; Portoghese, P. S. Clinically employed opioid analgesics produce antinociception via μ - δ opioid receptor heteromers in rhesus monkeys. *ACS Chem. Neurosci.* **2012**, *3*, 720–727.
- (60) Hasbi, A.; Nguyen, T.; Fan, T.; Cheng, R.; Rashid, A.; Alijaniam, M.; Rasenick, M. M.; O'Dowd, B. F.; George, S. R. Trafficking of preassembled opioid mu-delta heterooligomer-Gz signaling complexes to the plasma membrane: coregulation by agonists. *Biochemistry* **2007**, *46* (45), 12997–3009.
- (61) Akgun, E.; Javed, M. I.; Lunzer, M. M.; Smeester, B. A.; Beitz, A. J.; Portoghese, P. S. Ligands that interact with putative MOR-mGluR5 heteromer produce potent antinociception in mice with inflammatory pain. *Proc. Nat. Acad. Sci. U. S. A.* **2013**, *110*, 11595–11599.
- (62) Milan-Lobo, L.; Whistler, J. L. Heteromerization of the μ - and δ -opioid receptors produces ligand-biased antagonism and alters μ -receptor trafficking. *J. Pharmacol. Exp. Ther.* **2011**, *337* (3), 868–875.
- (63) Rozenfeld, R.; Devi, L. A. Receptor heterodimerization leads to a switch in signaling: β -arrestin2-mediated ERK activation by μ - δ opioid receptor heterodimers. *FASEB J.* **2007**, *21*, 2455–2465.
- (64) Gomes, I.; Fujita, W.; Gupta, A.; Saldanha, A. S.; Negri, A.; Pinello, C. E.; Roberts, E.; Filizola, M.; Hodder, P.; Devi, L. A. Identification of a μ - δ opioid receptor heteromer-biased agonist with antinociceptive activity. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (29), 12072–12077.
- (65) Wade, P. R.; Palmer, J. M.; McKenney, S.; Kenigs, V.; Chevalier, K.; Moore, B. A.; Mabus, J. R.; Saunders, P. R.; Wallace, N. H.; Schneider, C. R.; Kimball, E. S.; Breslin, H. J.; He, W.; Hornby, P. J. Modulation of gastrointestinal function by MuDelta, a mixed μ opioid receptor agonist/ μ opioid receptor antagonist. *Br. J. Pharmacol.* **2012**, *167* (5), 1111–1125.
- (66) Beslin, H. J.; Diamond, C. J.; Kavash, R. W.; Cai, C.; Dyatkin, A. B.; Miskowski, T. A.; Zhang, S. P.; Wade, P. R.; Hornby, P. J.; He, W. Identification of a dual δ OR antagonist/ μ OR agonist as a potential therapeutic for diarrhea-predominant irritable bowel syndrome (IBS-d). *Bioorg. Med. Chem. Lett.* **2012**, *22* (14), 4869–4872.
- (67) Milligan, G. A day in the life of a G protein-coupled receptor: the contribution to function of G protein-coupled receptor dimerization. *Br. J. Pharmacol.* **2008**, *153* (Suppl 1), 216–229.
- (68) Bulenger, S.; Marullo, S.; Bouvier, M. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol. Sci.* **2005**, *26*, 131–137.
- (69) Christopoulos, A.; Kenakin, T. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002**, *54* (2), 323–374.
- (70) Agnati, L. F.; Guidolin, D.; Albertin, G.; Trivello, E.; Ciruela, F.; Genedani, S.; Tarakanov, A.; Fuxe, K. An integrated view on the role of receptor mosaics at perisynaptic level: focus on adenosine A(2A), dopamine D(2), cannabinoid CB(1), and metabotropic glutamate mGlu(5) receptors. *J. Recept. Signal Transduction* **2010**, *30* (5), 355–369.
- (71) Zhang, Y. Q.; Limbird, L. E. Hetero-oligomers of alpha2A-adrenergic and mu-opioid receptors do not lead to transactivation of G proteins or altered endocytosis profiles. *Biochem. Soc. Trans.* **2004**, *32*, 856–860.
- (72) Pfeiffer, M.; Koch, T.; Schroder, H.; Laugsch, M.; Holtt, V.; Schulz, S. Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. *J. Biol. Chem.* **2002**, *277*, 19762–19772.
- (73) Jordan, B. A.; Trapaidze, N.; Gomes, I.; Nivarthi, R.; Devi, L. A. Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 343–348.
- (74) Javitch, J. A. The ants go marching two by two: oligomeric structure of G-protein-coupled receptors. *Mol. Pharmacol.* **2004**, *66* (5), 1077–1082.
- (75) Prinster, S. C.; Hague, C.; Hall, R. A. Heterodimerization of G protein-coupled receptors: specificity and functional significance. *Eur. J. Pharmacol.* **2005**, *57*, 289–298.
- (76) Mackie, K. Cannabinoid receptor homo- and heterodimerization. *Life Sci.* **2005**, *77*, 1667–1673.
- (77) Schroder, H.; Wu, D. F.; Seifert, A.; Rankovic, M.; Schulz, S.; Holtt, V.; Koch, T. Allosteric modulation of metabotropic glutamate receptor 5 affects phosphorylation, internalization, and desensitization of the micro-opioid receptor. *Neuropharmacology* **2009**, *56*, 768–778.