

Induced Association of μ Opioid (MOP) and Type 2 Cholecystokinin (CCK₂) Receptors by Novel Bivalent Ligands

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Both μ -opioid (MOP) and type 2 cholecystokinin (CCK₂) receptors are present in areas of the central nervous system that are involved in modulation of pain processing. We conducted bioluminescence resonance energy transfer (BRET) studies on COS cells coexpressing MOP and CCK₂ receptors to determine whether receptor heterodimerization is involved in such modulation. These studies revealed the absence of constitutive or monovalent ligand-induced heterodimerization. Heterodimerization of MOP and CCK₂ receptors therefore is unlikely to be responsible for the opposing effects between morphine and CCK in the CNS. However, association was induced, as indicated by a positive BRET signal, on exposure of the cells to bivalent ligands containing μ -opioid agonist and CCK₂ receptor antagonist pharmacophores linked through spacers containing 16–22 atoms but not with a shorter (9-atom) spacer. These studies demonstrate for the first time that an appropriately designed bivalent ligand is capable of inducing association of G-protein-coupled receptors. The finding that opioid tolerance studies with these ligands in mice showed no correlation with the BRET data is consistent with the absence of association of MOP and CCK₂ receptors *in vivo*.

Introduction

Both opioid (MOP^a) and cholecystokinin (CCK) receptors are members of the family-A G-protein-coupled receptor (GPCR) superfamily. Opioid receptors constitute a family of four receptor types, namely, MOP (μ), DOP (δ), KOP (κ), and NOP (nociceptin), having ~60% sequence homology. They are present in the central nervous system (CNS) and peripherally. Interaction of members of the opioid peptide family with opioid receptors triggers activation mainly of G_i or G_o, which mediate neuro- or immunomodulation.^{1,2}

There are two types of CCK receptors,³ CCK₁ and CCK₂, having sequence homology of approximately 50%.^{4–8} CCK₁ receptors are distributed principally in the peripheral alimentary tract, while CCK₂ receptors are found mainly in the CNS.⁹ Activation of both CCK receptors appears to be coupled primarily to G_q, resulting in the stimulation of phospholipase C and intracellular calcium, which leads to stimulation of gallbladder contraction, pancreatic exocrine secretion, enteric motility, gastric acid secretion, and modulation of neurotransmission.^{3–9}

Areas of the CNS that are involved in pain processing contain overlapping populations of CCK₂ and opioid receptors.^{10–12}

Activation of MOP or DOP receptors promotes the release of CCK which in turn acts to reduce opioid receptor-mediated analgesia through interaction with the CCK₂ receptor. CCK₂ receptor knockout mice display enhanced opioid-induced analgesia and up-regulation of the endogenous opioid receptor system.^{13,14} The rostral ventromedial medulla (RVM) is the key area where this effect takes place, and in this regard, increased CCK activity in the RVM during exposure to morphine leads to morphine tolerance.^{15,16}

Since antagonism of CCK₂ receptors is known to block morphine tolerance, the combined use of a CCK₂ antagonist and opioid agonist represents a potentially important strategy for the treatment of chronic pain states.¹⁷ In this regard, given the overlapping distribution of MOP and CCK₂ receptors in the brain, an important question concerning the opposing roles of these receptors relates to their physical organization, particularly in view of the relatively large number of opioid receptor heterodimers that are already known to exist.^{18–20}

We have investigated the possible existence of heterodimeric MOP/CCK₂ receptors through the careful application of bioluminescence resonance energy transfer (BRET) technology in cultured cells that contain both MOP and CCK₂ receptors. Our BRET data indicate the absence of constitutive or monovalent ligand-stimulated association of MOP and CCK₂ receptors. However, in the presence of bivalent ligands that contain a μ -opioid agonist pharmacophore linked through an appropriate length spacer to a CCK₂ antagonist pharmacophore, the association of MOP and CCK₂ receptors was observed.

Design Considerations for Bivalent Ligands

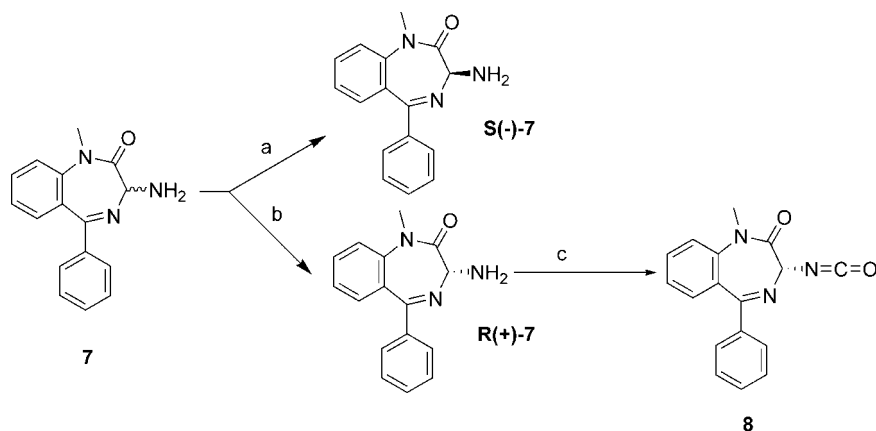
The design approach for the bivalent ligands **3a–c** and **4** (Figure 1) employed in this study consisted of the tethering of a μ -opioid agonist pharmacophore with a CCK₂ antagonist pharmacophore using a spacer. Nonpeptidic pharmacophores were employed because it has been established that they bind within the cavity of the heptahelical (seven transmembrane

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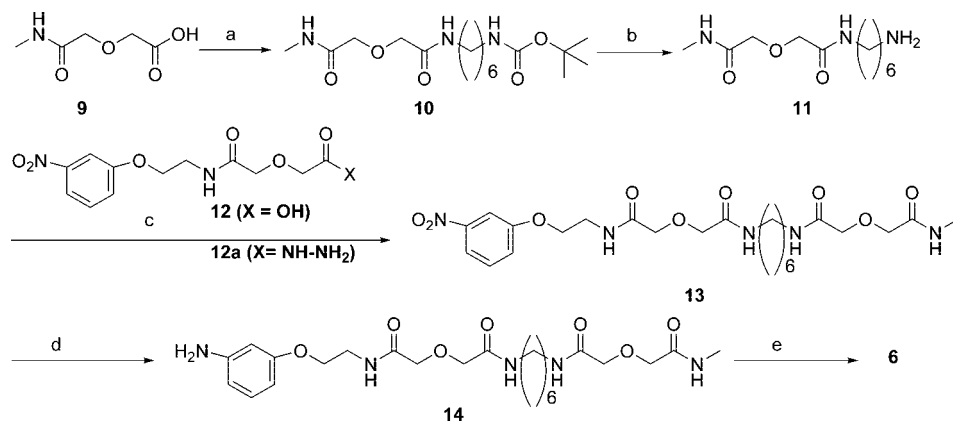
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^a Abbreviations: BRET, bioluminescence energy transfer; CNS, central nervous system; CCK, cholecystokinin; CCK₁R, cholecystokinin-1 receptor; CCK₂R, cholecystokinin-2 receptor; DCC, *N,N'*-dicyclohexylcarbodiimide; CDCl₃, chloroform-*d*; CHCl₃, chloroform; CH₂Cl₂, dichloromethane; DAM-GO, [D-Ala², NMePhe⁴, Gly⁵-ol]enkephalin; DMEM, Dulbecco's modified Eagle's medium; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DOP, δ -opioid peptide; ESI, electrospray ionization; EtOAc, ethyl acetate; FLIPR, fluorometric imaging plate reader; GPCR, G-protein-coupled receptor; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; KOP, κ -opioid peptide; MeOH, methanol; MOP, μ -opioid; MOPR, μ -opioid receptor; NOP, nociceptin; Pd/C, palladium on carbon; Rlu, Renilla luciferase; RVM, rostral ventromedial medulla; THF, tetrahydrofuran; TFA, trifluoroacetic acid; YFP, yellow fluorescent protein.

Scheme 1^a

^a (a) (i) (1*S*)-(+)-10-camphorsulfonic acid, CH₃CN/diethylether; (ii) 10% NaOH, CH₂Cl₂; (b) mother liquor from first step taken; (i) (1*R*)-(-)-10-camphorsulfonic acid, CH₃CN/diethyl ether; (ii) 10% NaOH, CH₂Cl₂; (c) triphosgene, aqueous saturated Na₂CO₃/CH₂Cl₂, 0 °C.

Scheme 2^a

^a (a) HOBt/DCC, DMF, Boc-hexadamine1,6, room temp; (b) TFA/CH₂Cl₂, room temp; (c) only **12** used, HOBt/DCC, DMF, room temp; (d) Pd/C (10%), methanol, 50 psi; (e) **8**, DMF, 5 days.

2-(3 nitrophenoxy)ethanolamine **21**³³ with the glycolic acid derivative **16**. The intermediate nitro compound **22** was reduced to amine **23** via catalytic hydrogenolysis in methanol using Pd/C (10%) at 50 psi. The reaction of **23** with **8** in DMSO gave bivalent ligand **4** (Scheme 4).

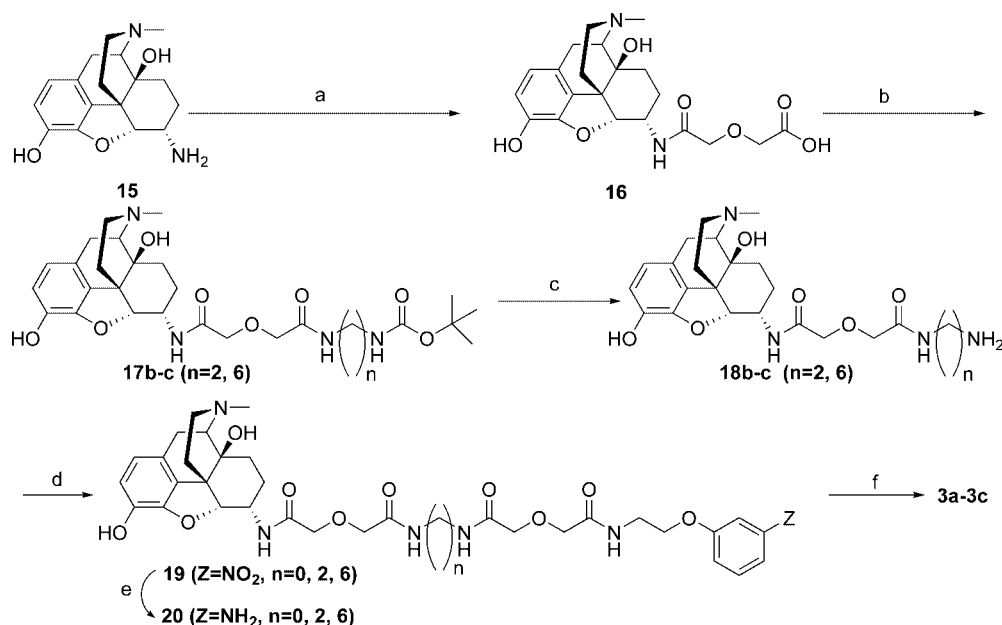
Biological Results

BRET Studies. BRET technology has been utilized previously to demonstrate that CCK₂ receptors exist as constitutive homodimers whose BRET signal is unaffected by the binding of endogenous agonist.³⁴ The literature supports similar constitutive homodimerization of MOP receptors.^{19,35} This was confirmed in control studies with cells expressing only MOP or CCK₂ receptors in that a positive homologous receptor BRET signal was observed (Figure 2). Background nonspecific BRET signals were determined by expressing the complementary fluorescent donor or acceptor in a soluble form or attached to a structurally unrelated family B GPCR, the secretin receptor.

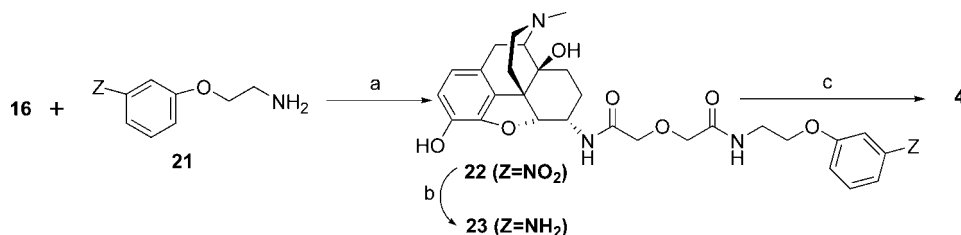
However, receptor association could be induced with bivalent ligands that were capable of bridging the CCK₂ and MOP receptors, as reflected by a positive heterologous receptor BRET signal (Figure 3). The top panels show that significant BRET signals were produced by **3a** (16-atom spacer), **3b** (18-atom spacer), and **3c** (22-atom spacer) while the spacer in **4** (9 atoms) is apparently too short to generate such a signal. The highest BRET signal was generated with the bivalent ligand **3b** having the 18-atom spacer. Competition between the monovalent CCK₂

antagonist **6** and **3a–c** was reflected by a reduction in the BRET ratio, as shown in the middle panel. The bottom panel illustrates that no BRET signal was produced with monovalent ligands **5** and **6** individually or together. Thus, heterodimerization of these receptors was not induced by the occupation of the receptors with ligands that do not include a linking domain. It is noteworthy that neither the BRET ratios of MOP or CCK₂ receptor homodimers were affected by either bivalent ligand **3c** or monovalent ligands **5** and **6** utilized in this work (Figure 4).

Saturation BRET assays were performed to distinguish specific molecular interactions from random interactions between donor and acceptor that can occur in a concentration-dependent manner. Figure 5 shows COS cells expressing Rlu-tagged MOP receptor and YFP-tagged CCK₂ receptor after transfection using a fixed amount of donor construct (1.0 μg DNA/dish) and increasing amounts of acceptor construct (0.3 μg to 6 μg DNA/dish). This produced the expected increase in BRET ratio that then reached a plateau for bivalent ligands **3b,c**. These data support the saturable nature of these molecular interactions. The plateau of the BRET signal was typically reached with 1.5–2.0 μg acceptor DNA/dish, representing approximate molar ratios of acceptor/donor of 1.5–2.0:1. This supports the specific interactions between MOP and CCK₂ receptors stimulated by the bivalent ligands that were suggested by the static BRET studies described above. The presence of the combination of monovalent ligands, **5** and **6**, or the absence

Scheme 3^a

^a (a) Diglycolic anhydride, CH₂Cl₂, room temp; (b) HOBt/DCC, mono-Boc-diamines ($n = 2$; $n = 6$), DMF; (c) TAF/CH₂Cl₂; (d) **12**, HOBt/DCC; (e) Pd/C (10%), methanol, 50 psi; (f) **8**, DMF, ~24 h, room temp.

Scheme 4^a

^a (a) HOBt/DCC, DMF; (b) Pd/C (10%), methanol, 50 psi; (c) **8**, DMSO, 24 h, room temp.

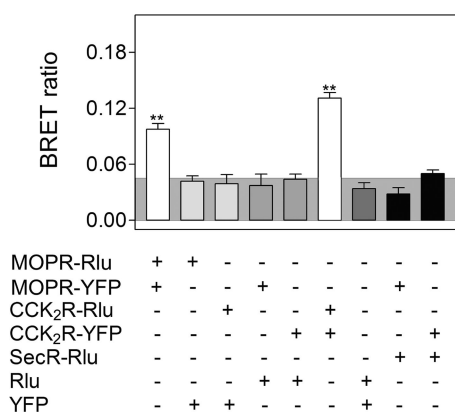


Figure 2. BRET controls. Shown are the BRET ratios for COS cells expressing various constructs in the combinations noted. The shaded area represents the intensity of BRET signals felt to be nonspecific, reflecting a signal that can be generated between Rlu-tagged receptor and soluble YFP protein or between YFP-tagged receptor and soluble Rlu protein. BRET signals above this are considered to be significant. Both CCK₂ receptors and MOP receptors exist as constitutive homodimers with significant homologous receptor BRET signals when expressed in these cells. Values presented represent the mean \pm SEM of data from five independent experiments. Values marked with ** represent BRET signals significantly above nonspecific values at the $p < 0.001$ level.

of ligand yielded only a low level signal that was not saturable, with best fit of the data not different from a linear regression.

These data are consistent with random interactions between these receptors taking place in the plasma membrane.

Binding studies of the bivalent ligands were conducted in singly expressed and coexpressed CHO cells. Only **3a** and **3b** showed significantly higher affinity for CCK₂ receptors in coexpressed cells relative to singly expressed cells, whereas little, if any, affinity differences for MOP receptors were observed between singly and doubly expressed cells. The reason for these results is not clear, but one possibility is that the conditions employed in binding interfered with association of CCK₂ and MOP receptors.

Given the results of the present study that demonstrate that MOP and CCK₂ receptors do not constitutively form heterodimers but can be induced to associate in the presence of appropriate bivalent ligands, we evaluated the functional properties of bivalent ligand **3b** and monovalent ligands **5** and **6** in CHO cells coexpressed with MOP and CCK₂ receptors using the calcium mobilization assay.³⁶ The data (Table 2) revealed that **3b** and the MOP receptor agonist **5** promoted Ca²⁺ release whereas the monovalent CCK antagonist **6** did not. Interestingly, both ligands were capable of activating opioid receptors, although **3b** was 4.8-fold more potent than monovalent agonist **5** in this regard. These studies suggest that opioid receptor activation can occur in the induced association state or as homomers.

Experiments in mice were conducted to evaluate the tolerance of the bivalent ligands with 9-, 16-, 18-, and 22-atom spacers

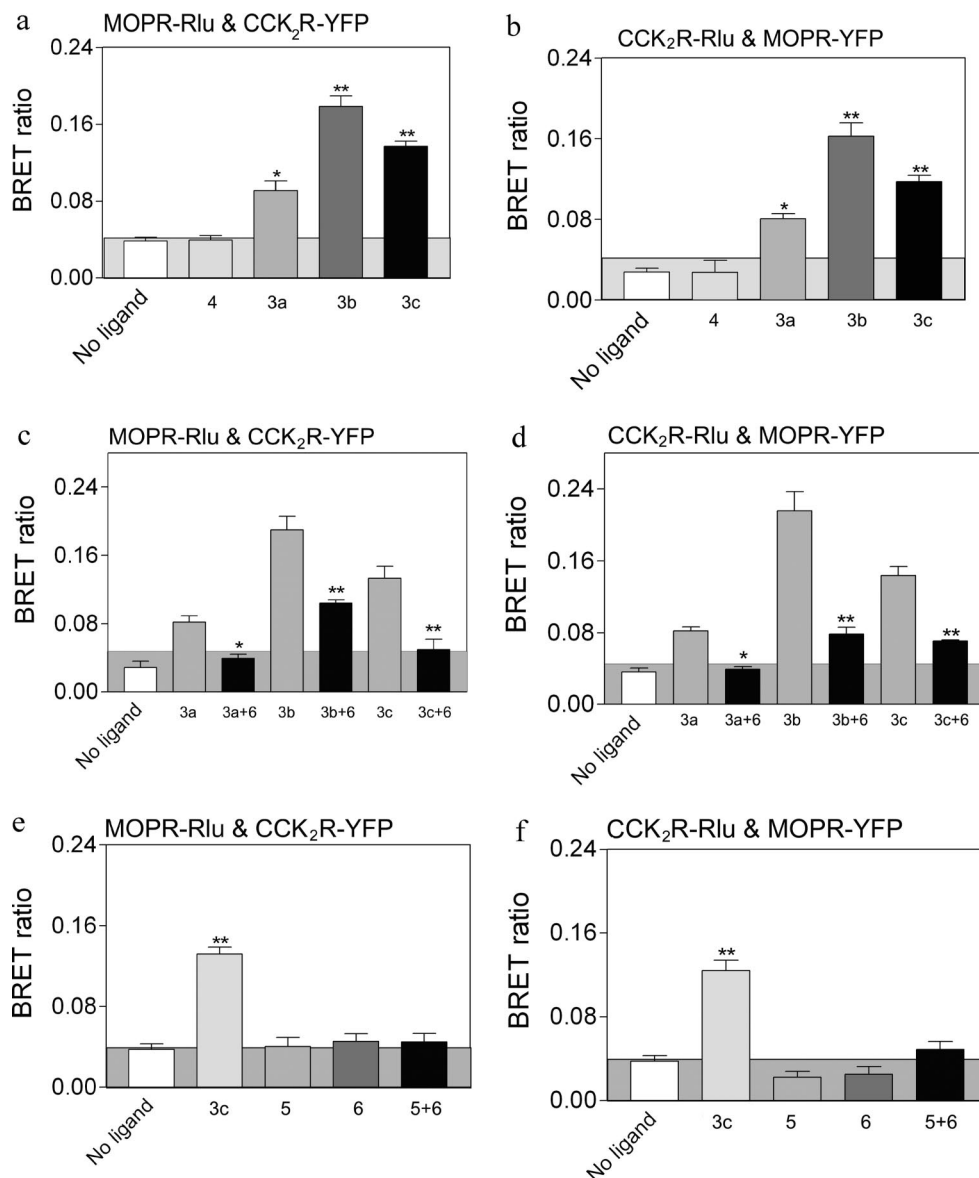


Figure 3. Effects of bivalent ligands on heterodimerization of CCK₂ and MOP receptors. Shown are BRET ratios for COS cells expressing both tagged MOP and CCK₂ receptor constructs with or without incubation of various bivalent ligands (10^{-6} M, 90 min at room temperature). The panels on the left reflect Rlu-tagged μ -opioid receptor expressed with YFP-tagged CCK₂ receptor, while those on the right represent the opposite. The panels in the top row illustrate BRET ratios for these cells in the absence of ligand and in the presence of bivalent ligands of various spacer lengths. The panels in the second row reflect the saturability of the BRET ratios in the presence of the bivalent ligands that normally give a significant signal, that are reduced in the presence of competing monovalent CCK antagonist ligand **6**. The panels in the third row demonstrate that monovalent ligands that recognize only the CCK₂ receptor or the opioid receptor, even when mixed together, failed to produce the BRET signal observed with the bivalent ligand. The shaded area represents the nonspecific BRET signal as described in Figure 2. Values represent the mean \pm SEM of data from five independent experiments: (*) $p < 0.05$; (**) $p < 0.001$.

4, **3a–c** relative to that of the control monovalent **5**. These data are summarized in Table 3. With the exception of **5**, none of the bivalents exhibited tolerance via the icv route. Given that bivalent ligand **4** with the shortest spacer did not show tolerance and the quantitative BRET data suggesting the absence of bridging between MOP and CCK₂ receptors for **4**, there appears to be no relationship between ligand-induced association of receptors and blockage of tolerance.

Discussion

Careful and systematic BRET analysis shows that MOP and CCK₂ receptors coexpressed in COS cells are present as homodimers, with no evidence of spontaneous receptor heterodimerization. Evidently, the association of either MOP receptors or CCK₂ receptors as homodimers is more favorable

than their association to heterodimers. However, association of these receptors could be induced in the presence of bivalent ligands **3a–c** that contain both μ -opioid agonist and CCK₂ receptor antagonist pharmacophores connected through spacers whose length is in the range of 16–22 atoms. In this regard, the BRET signal for **3b,c** plateaued close to a BRET ratio of ~ 2 , supporting specific saturable interaction between MOP and CCK₂ receptors that was suggested by the BRET ratio enhancement. It is noteworthy that the BRET ratio plateau for **3a** was approximately half that of **3b** and **3c**. This could reflect the low efficiency of induction of association by this ligand and different alignment of donor and acceptor fluorophores attached to their receptors as a consequence of this relatively short spacer. Significantly, the bivalent ligand **4** with the shortest spacer (9 atoms) as well as the simultaneous use of the monovalent ligands

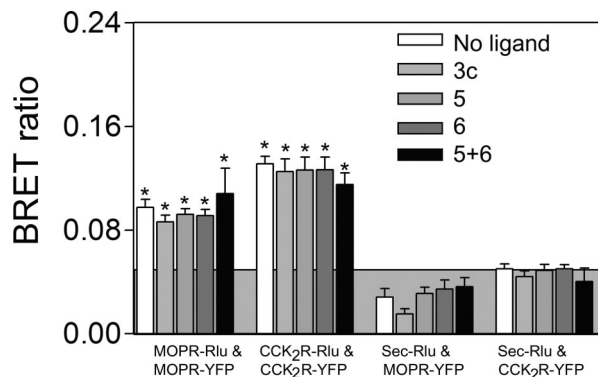


Figure 4. Effects of ligands on receptor homodimerization. Shown are the BRET signals stimulated by bivalent or monovalent ligands in COS cells expressing Rlu- and YFP-tagged CCK₂ receptor constructs or MOP receptor constructs or structurally unrelated tagged receptor constructs. None of the ligands studied had any effect on the constitutive homodimers of the CCK₂ or MOP receptors and did not lead to heterodimerization of the unrelated receptors. Values represent the mean \pm SEM of data from six independent experiments: (*) $p < 0.05$.

5 and **6** did not induce any recognizable association of MOP and CCK₂ receptors. These data suggest that only bivalent ligands **3a–c** were capable of inducing association because the spacers are of sufficient length to permit binding of each of the pharmacophores to their respective receptors through a bridging mechanism.

The association of MOP and CCK₂ receptors induced by **3a–c**, as reflected by the heterologous receptor BRET ratios, appears to be most favorable with **3b** which contains the 18-atom spacer. The finding that no significant difference of BRET ratio was observed with the monovalent ligands **5**, **6**, or bivalent ligand **4** in cells that contain either homodimerized MOP or CCK₂ receptors indicates that these ligands do not affect the degree of constitutive homodimerization.

In the presence of the CCK₂ receptor monovalent antagonist ligand **6**, the BRET ratios of bivalent ligands **3a–c** were reduced. These data suggest that competition of **6** with the CCK₂ receptor antagonist pharmacophore of the bivalent ligands involved in bridging associated MOP/CCK₂ receptors promotes a transition from the bridged binding mode to univalent binding. In the absence of constraints due to bridging, the heteromers dissociate and form the more stable homodimers.

On the basis of the present work, a schematic for induction of association of MOP and CCK₂ receptors is illustrated in Figure 6. Thus, GPCRs that do not easily associate to form heterodimers can be associated using an appropriately designed bivalent ligand. On the other hand, if coexpressed GPCRs exhibit greater ability to associate as heterodimers, as opposed to homodimers, it might be expected that they would exist as constitutive heterodimers. In this connection, the presence of constitutive heterodimers in cultured cells has been reported for a number of opioid^{37–39} and CCK⁴⁰ receptors. Of significance is the finding that interacting ligands that selectively target or activate putative heterodimers *in vivo* may have pharmacological profiles that differ from those that interact with homodimers.³⁰

Because our results in cultured cells indicated the absence of constitutive association in MOP and CCK receptors (in contrast to literature⁴¹ which has reported numerous heterodimeric opioid receptors in cultured cells), thereby making it unlikely that this may be a mechanism for the modulation of morphine tolerance and potency, we decided to determine the *icv* ED₅₀ values of the series in order to evaluate whether or

not our ligands produce tolerance. The *in vivo* data (Table 3) revealed that only monovalent ligand **5** produced tolerance, either alone or in equimolar combination with monovalent CCK₂ antagonist **6**. On the other hand, none of the bivalent ligands **3a–c**, **4** gave rise to significant tolerance, despite our finding that **4** does not induce association of MOP and CCK₂ receptors in COS cells. Given our evidence for lack of constitutive association of MOP and CCK₂ receptors in COS cells, it would be expected that **4** would produce tolerance in mice if a heterodimer were involved in the modulation process. When taken together with the results of the BRET data, the tolerance studies therefore suggest that the modulation of morphine's effects is possibly due to circuitry rather than to physical association of MOP and CCK₂ receptors as heterodimers.

In conclusion, this study demonstrates proof of principle concerning the ability of bivalent ligands to induce association of receptors through a bridging mechanism. In this regard, it has not escaped our attention that other bivalent ligands^{21–25} whose pharmacologic profiles suggest bridging of dimeric opioid receptors have spacer lengths in the same range as the effective bivalent ligands in the present series. We believe this similarity may reflect a common structural organization of GPCR dimers. Thus, pharmacophores that bind within the helical bundle and are linked by linear spacers greater than 16 atoms in length with optimal lengths in the range of 18–21 atoms have been reported to most effectively bridge dimeric GPCRs.^{21–25}

Experimental Section

Chemistry. General. Oxymorphone was obtained from Mallinckrodt & Co. All other chemicals and solvents were purchased from Aldrich or Fisher without further purification. Some dry solvents were made by MBRAUN MB-SPS Solvent Dispensing.

¹H and ¹³C NMR spectroscopy were obtained on 300 MHz on an Oxford Varian VXR 300 MHz NMR spectrometer. Polarities were obtained on AUTOPOL III automatic polarimeter. Mass spectroscopy was obtained on Bruker BioTOF II mass spectrometer.

Purities of the monovalent ligands **6** and bivalent ligands **3a–c** and **4** were over 98% based on analysis on HPLC column (Phenomenex Luna SB-C18 (2) 5 μ m, 4.6 mm \times 250 mm) which was eluted with MeOH/buffer (60:40) at a flow rate of 1 mL/min.

3(S)-(–)-Amino-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (S(–)-7). A solution of **7** (1.0 g, 3.9 mmol) in acetonitrile (4 mL) was treated with 1(S)-(+)-camphorsulfonic acid (0.91 g, 3.9 mmol) and diethyl ether (12 mL) and aged overnight at room temperature. The crystals were filtered and washed with acetonitrile/ether (1:3), yielding 0.92 g of solid as 3(S)-(–)-amine-1(S)-(+)-camphorsulfonate salt (98.1%). [α]_D²² –88.

The salt (920 mg) was treated with 10% NaOH (6 mL) in CH₂Cl₂ (6 mL). The phases were separated, and the aqueous layer was extracted with CH₂Cl₂ (3 mL \times 5). The organic layers were combined, dried over MgSO₄, and concentrated by a rotary-evaporator to yield 459.4 mg (89.8%) of 3(S)-(–)-amine. [α]_D²² –250, [α]_D²⁵ –269.³¹ ¹H NMR (CDCl₃): δ 7.62–7.69 (m, ArH, 2H), 7.55–7.53 (m, ArH, 1H), 7.43–7.29 (m, ArH, 5H), 7.21–7.16 (m, ArH, 1H), 4.46 (s, CH, 1H), 3.45 (s, CH₃, 3H), 2.75 (s, NH₂, 2H). ¹³C NMR (CDCl₃): δ 170.14, 165.85, 142.97, 138.03, 131.46, 130.29, 130.01, 129.50 (\times 2), 129.04, 128.07 (\times 2), 123.87, 121.14, 70.27, 35.27.

3(R)-(+)-Amino-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (R(+)-7). The mother liquor from resolution of **7** with 1(S)-(+)-camphorsulfonic acid was concentrated by a rotary-evaporator. The residual was treated with 10% NaOH (30 mL) in CH₂Cl₂ (30 mL) and extracted with CH₂Cl₂ (10 mL \times 5). The extracts were combined and concentrated by a rotary-evaporator. The residual was dissolved in acetonitrile (2.2 mL) and treated with 1(R)-(+)-10-camphorsulfonic acid (0.49 g, 2.1 mmol) and diethyl ether (6.6 mL). The mixture was stored overnight at room temperature and the crystals were filtered and washed with

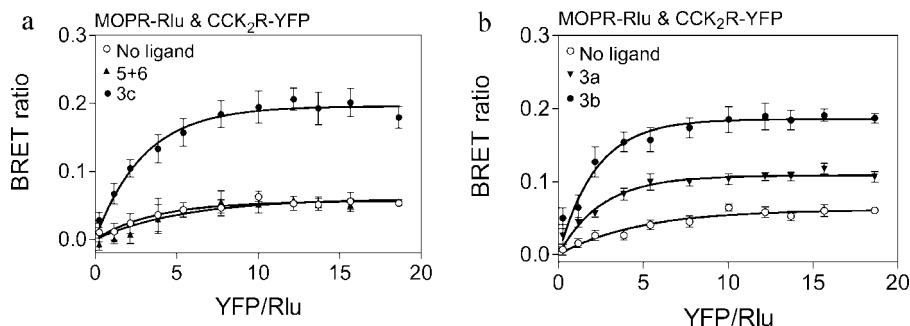


Figure 5. Effects of bivalent ligands on bystander BRET. Shown are the saturation curves obtained by coexpressing pairs of Rlu-tagged MOP receptor and YFP-tagged CCK₂ receptor constructs after incubation with bivalent ligands (10⁻⁶ M), as indicated. Bivalent ligands **3a–c** produced exponential curves that reached a plateau, supporting specific molecular interactions. Cells treated with no ligand or with a combination of monovalent ligands for the CCK and μ -opioid receptors showed curves not significantly different from linear fits. Values represent the mean \pm SEM of data from five independent experiments.

Table 1. Binding of DAMGO, **2**, Bivalent Ligands **3a–c**, and Monovalent Ligands **4** and **6** to Singly and Coexpressed CCK₂ and MOP Receptors in CHO Cells^a

ligands	[¹²⁵ I]CCK		[³ H]DAMGO	
	K _i (nM)	B _{max} (pmol/(mg of protein))	K _i (nM)	B _{max} (pmol/(mg of protein))
Cells Expressing CCK ₂ Receptor				
2	18 \pm 3	14.4 \pm 3.4		
3a	356 \pm 67	16.0 \pm 3.7		
3b	71 \pm 5	10.3 \pm 1.8		
3c	260 \pm 90	18.5 \pm 1.7		
4	223 \pm 11	14.0 \pm 3.9		
6	48 \pm 6	16.0 \pm 1.1		
Cells Expressing MOP Receptor				
DAMGO			6.7 \pm 2.9	9.2 \pm 2.7
3a			8.3 \pm 2.4	9.8 \pm 3.5
3b			5.7 \pm 1.7	7.7 \pm 1.9
3c			29.4 \pm 2.1	7.4 \pm 0.5
4			24.1 \pm 0.7	12.4 \pm 1.9
5			29.8 \pm 10.2	9.3 \pm 1.4
Cells Expressing CCK ₂ Receptor/MOP Receptor				
DAMGO			11.0 \pm 6.2	9.5 \pm 3.3
2	14 \pm 2	9.1 \pm 1.7		
3a	57 \pm 10	9.5 \pm 1.9	3.9 \pm 2.1	8.3 \pm 1.4
3b	33 \pm 3	12.4 \pm 1.8	7.3 \pm 1.5	9.7 \pm 2.5
3c	77 \pm 11	10.9 \pm 2.8	16.3 \pm 0.5	9.3 \pm 1.4
4	210 \pm 44	9.3 \pm 2.5	14.2 \pm 2.5	10.5 \pm 12.6
5			40.7 \pm 9.5	7.4 \pm 0.6
6	21 \pm 3	9.5 \pm 3.9		

^a Values are expressed as the mean \pm SEM of data from three to five independent experiments.

Table 2. G_{aqi4myr}-Mediated Ca²⁺ Release of CHO Cells Stably Expressing MOP and CCK₂ Receptors

	EC ₅₀ (nM)			
	3b	5	6	DAMGO
MOPR-CCK ₂ R CHO	119 \pm 75 (4)	572 \pm 71.1(3)	<10000 (3)	2.66 \pm 0.70 (4)

Table 3. Comparison of the ED₅₀ Values (pmol/Mouse) of Bivalent Ligands **3a–c** and **4**, Monovalent Ligand **5**, and Simultaneous Administration of Monovalent Ligands **5** and **6**: A 24 h Tolerance Study

	ED ₅₀ (pmol/mouse)					
	3a	3b	3c	4	5	5 + 6
icv	47.41 (37.19–60.44)	390 (280–530)	2020 (1420–2870)	3036 (2390–3857)	51.93 (39.00–69.14)	96.29 (64.93–142.8)
control ^a	83.86 \pm 8.76	80.67 \pm 9.79	83.93 \pm 8.05	75.45 \pm 9.84	70.61 \pm 9.65	82.04 \pm 12.08
24 h ^{b,c}	84.45 \pm 7.81	71.29 \pm 8.55	64.33 \pm 13.10	57.31 \pm 12.98	40.74 \pm 11.86 ^d	42.94 \pm 13.40 ^d

^a The control value represents the ED₈₀ dose for each individual agonist on day 1. ^b Twenty-four hours later the mice were first tested to see that the percent antinociception was back to baseline and then the ED₈₀ dose was tested to see if there was any tolerance. ^c Peak time is between 10 and 20 min.

^d Tolerance formed.

cold acetonitrile/ether (1:3), yielding 0.81 g (86.4%) of solid as 3(R)-(+)-amine-1(R)-(-)-camphorsulfonate salt. [α]_D²⁵ +88.

The salt (810 mg) was treated with 10% NaOH (6 mL) in CH₂Cl₂ (6 mL). The phases were separated, and the aqueous layer was

extracted with CH₂Cl₂ (3 mL \times 5). The organic layers were combined, dried over MgSO₄, and concentrated in a rotary-evaporator to yield 406 mg (97.3%) of 3(R)-(+)-amine. [α]_D²⁵ +262. ¹H NMR (CDCl₃): δ 7.62–7.60 (m, ArH, 2H), 7.56–7.53 (m, ArH,

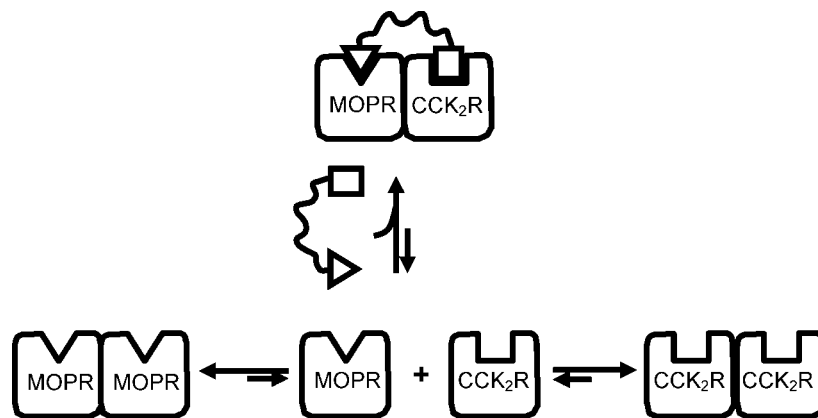


Figure 6. Schematic illustration of the effect of a MOP agonist/CCK₂ antagonist bivalent ligand on the equilibrium between a mixture of MOP receptor (MOPR) and cholecystokinin receptors (CCK₂R). Note that the bivalent ligand induces heterodimerization through constraint imposed by its spacer.

1H), 7.44–7.30 (m, ArH, 5H), 7.21–7.17 (m, ArH, 1H), 4.46 (s, CH, 1H), 3.45 (s, CH₃, 3H), 2.61 (s, NH₂, 2H). ¹³C NMR (CDCl₃): δ 170.20, 165.85, 143.01, 138.07, 131.49, 130.31, 130.03, 129.52 (×2), 129.08, 128.10 (×2), 123.89, 121.16, 70.32, 35.30.

1,3-Dihydro-3(R)-(+)-isocyanate-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (8). To the biphasic mixture of *R*(+)-7 (600 mg, 2.26 mmol) in CH₂Cl₂ (10 mL)/NaHCO₃ (saturated aqueous solution) cooled in an ice bath was added triphosgene (224 mg, 0.753 mmol). The reaction mixture was stirred in the ice bath for 20 min and then poured into a separator funnel. The organic layer was collected, and the aqueous layer was extracted with CH₂Cl₂ (3 × 1.5 mL). The combined organic layers were dried over MgSO₄ and concentrated in vacuum to give 553.9 mg (84.1%) of **8**. ¹H NMR (CDCl₃): δ 7.67–7.4 (m, ArH, 9H), 4.94 (m, CH, 1H), 3.52 (s, CH₃, 3H). MS 314.21 (M⁺ + Na).

***N*-Methyldiglycolamide (9).** To a cold (0 °C) 2 M solution of methylamine in THF (10 mL, 20 mmol) was added commercially available diglycolic anhydride (2.32 g, 20 mmol). The reaction solution was stirred overnight at room temperature and concentrated by a rotary-evaporator. The residue was dried by a vacuum pump without further purification to give 2.82 g (95.8%) as an oil. ¹H NMR (DMSO-*d*₆): δ 12.76 (s, CO₂H, 1H), 7.78 (s, NH, 1H), 4.07 (s, CH₂, 2H), 3.93 (s, CH₂, 2H), 2.61 (s, CH₃, 3H). ¹³C NMR (DMSO-*d*₆): δ 171.23, 168.95, 70.08, 67.75, 25.23.

***N*-(BOC-hexamethylamino)-*N'*-methyldiglycoldiamide (10).** To a solution (0 °C) of **9** (0.37 g, 2.5 mmol), DCC (0.57 g, 2.75 mmol), and HOBt (0.37 g, 2.75 mmol) in dry DMF (2 mL) was added a solution of mono-Boc protected hexamethylene amine (1,6) (0.54 g, 2.5 mmol) in DMF (1.5 mL). The reaction mixture was stirred for 2 days at room temperature, and then the precipitate was filtered. The filtrate was concentrated and the residue was purified by flash chromatography (CH₃OH/CH₂Cl₂/NH₃H₂O = 5:95:0.5) to give 0.42 g (61.0%) of **10**. ¹H NMR (CDCl₃): δ 6.91 (s, CONH, 2H), 4.61 (s, CONH, 1H), 4.05 (s, diglCH₂, 2H), 4.04 (s, diglCH₂, 2H), 3.29 (m, hexCH₂, 2H), 3.14 (m, hexCH₂, 2H), 2.85 (m, CH₃, 3H), 1.57–1.34 (m, hexCH₂, 8H), 1.44 (s, CH₃, 9H). MS 346.28 (M⁺ + H), 368.27 (M⁺ + Na).

***N*-(Aminomethyl)-2-(2-(methylamino)-2-oxoethoxy)-acetamide (11).** *N*-(BOC-hexamethylamino)-*N'*-methyldiglycoldiamide **10** (0.4 g, 1.16 mmol) was dissolved in TFA/CH₂Cl₂ (1:1, 20 mL) and stirred at room temperature until no more starting materials could be detected on TLC. Solvent was evaporated and the residue was purified by flash chromatography (CH₃OH/CH₂Cl₂/NH₄OH) to give 100 mg (35.1%) of **11**. ¹H NMR (DMSO-*d*₆): δ 8.03 (m, CONH, 2H), 7.71 (s br, CONH, 1H), 7.16 (t br, NH₂, 2H), 3.89 (s, diglCH₂, 4H), 3.09 (m, hexCH₂, 2H), 2.75 (m, hexCH₂, 2H), 2.63 (d, *J* = 7.8 Hz, CH₃, 3H), 1.50 (m, hexCH₂, 2H), 1.41 (m, hexCH₂, 2H), 1.26 (m, hexCH₂, 4H). MS 246.20 (M⁺ + H), 268.19 (M⁺ + Na).

2-(2-(3-Nitrophenoxyamino)-2-oxoethoxy)acetic Acid (12). To a suspension of diglycolic anhydride (5.8 g, 50 mmol) in dry CH₂Cl₂

(100 mL) was added a solution of commercially available 2-(3-nitrophenoxy)ethanamine (7.6 g, 55 mmol) in dry THF (100 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight. Filtration of the precipitate gave 10.97 g (86.3%) of **12**. ¹H NMR (DMSO-*d*₆): δ 12.87 (s br, CO₂H, 1H), 10.37 (s, CONH, 1H), 8.67 (m, ArH, 1H), 8.01 (m, ArH, 1H), 7.92 (m, ArH, 1H), 7.61 (m, ArH, 1H), 4.22 (s, CH₂, 4H), 4.21 (s, CH₂, 4H). ¹³C NMR (DMSO-*d*₆): δ 171.53, 168.56, 147.75, 139.40, 130.03, 125.51, 118.02, 113.55, 70.22, 67.97.

2-(2-Hydrazinyl-2-oxoethoxy)-*N*-(2(3nitrophenoxy)ethyl)-acetamide (12a). To a solution of **12** (149 mg, 0.5 mmol) in anhydrous DMF (1.5 mL) were added DCC (113.5 mg, 0.55 mmol), HOBt (148.6 mg, 1.1 mmol), and hydrazine hydrate (29 μL, 0.6 mmol) at room temperature. The reaction mixture was stirred 24 h at room temperature, and the precipitate was removed by filtration. The filtrate was concentrated and the residual was purified by flash chromatography (CH₂Cl₂/MeOH/NH₄OH = 85:14.5:0.5) and gave 54 mg (34.6%) of **12a**. ¹H NMR (CDCl₃): δ 7.86 (dd, *J* = 8.1, 2.1 Hz, 1H), 7.75 (dd, *J* = 2.1, 2.1 Hz, 1H), 7.68 (s, 1H), 7.46 (dd, *J* = 8.1, 8.1 Hz, 1H), 7.27 (dd, *J* = 8.1, 21 Hz, 1H), 6.95 (m, 1H), 4.17 (t, *J* = 5.4, 2H), 4.15 (s, 2H), 4.09 (s, 2H), 3.78 (dt, *J* = 5.4, 2H), 1.72 (s br, 2H). MS 335.32 (M⁺ + Na).

***N*-Methyl-2-(13-(3-nitrophenoxy)-2,6,10-trioxo-8-oxa-3,5,11-triazatridecyloxy)acetamide (13).** To a solution of **12** (0.714 g, 2.81 mmol), DCC (0.58 g, 2.81 mmol), and HOBt (0.38 g, 2.81 mmol) in dry DMF (2.5 mL) was added a 2.5 mL dry DMF solution of **11** (0.625 g, 2.55 mmol). The reaction mixture was stirred overnight at room temperature, and the precipitate was filtered. The filtrate was concentrated in a rotary-evaporator and purified by flash chromatography (CH₂Cl₂/CH₃OH/NH₃H₂O = 90:9.5:0.5) to give 0.48 g (39.1%) of **13**. ¹H NMR (DMSO-*d*₆): δ 10.53 (s, CONH, 1H), 8.67 (m, CONH, 1H), 8.08 (m, ArH, 1H), 8.00 (m, ArH + CONH, 3H), 7.94 (m, ArH, 1H), 7.62 (m, ArH, 1H), 4.18 (s, CH₂, 2H), 4.05 (s, CH₂, 2H), 3.89 (s, CH₂, 4H), 3.10 (m, CH₂, 4H), 2.63 (d, *J* = 4.8 Hz, CH₃, 3H), 1.42 (m, CH₂, 4H), 1.25 (m, CH₂, 4H). MS 482.4 (M⁺ + H), 503.3 (M⁺ + Na).

2-(13-(3-Aminophenoxy)-2,6,10-trioxo-8-oxa-3,5,11-triazatridecyloxy)-*N*-methylacetamide (14). A mixture of **13** (90 mg, 0.171 mmol) and Pd/C of 10 wt % (30 mg) in MeOH (5 mL) was hydrogenated at 50 psi for 4 h at room temperature. The catalyst was removed by filtration over a Celite pad, and the filtrate was concentrated by a rotary-evaporator to give 75 mg (88.5%) of product **14**. ¹H NMR (DMSO-*d*₆): δ 8.21 (m, CONH, 1H), 8.01 (m, CONH, 3H), 6.86 (t, *J* = 8.1 Hz, ArH, 1H), 6.14–6.11 (m, ArH, 2H), 6.06 (m, ArH, 1H), 5.02 (m, NH₂, 1H), 3.94 (s, CH₂, 2H), 3.91 (s, CH₂, 2H), 3.89 (m, CH₂, 6H), 3.45 (m, CH₂, 2H), 3.09 (m, CH₂, 4H), 2.63 (d, *J* = 4.5 Hz, CH₃, 3H), 1.41 (m, CH₂, 4H), 1.24 (m, CH₂, 4H). MS 496.61 (M⁺ + H), 518.59 (M⁺ + Na).

(*R*)-*Z*-*N*-Methyl-2-(13-(3-(3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[*e*][1,4]diazepin-3-yl)ureido)phenoxy)-

2,6,10-trioxo-8-oxa-3,5,11-triazatridecyloxyacetamide (6). To a solution of **14** (75 mg, 0.151 mmol) and isocyanate derivative **8** (71.7 mg, 0.166 mmol) in anhydrous DMF (4 mL) was added triethylamine (23.2 μ L, 0.166 mmol). The reaction solution was stirred at room temperature for 5 days and concentrated. The residue was purified by flash chromatography to give 23 mg (19.4) of product **6**. ¹H NMR (CDCl₃): δ 8.11 (m, CONH, 1H), 7.59 (m, CONH, 3H), 7.37 (m, ArH, 5H), 7.17–6.91 (m, ArH, 6H), 6.52 (m, CONH, 1H), 5.49 (d, J = 7.8 Hz, CH, 1H), 5.02 (m, CONH, 1H), 4.04 (s, CH₂, 2H), 4.01 (m, CH₂, 6H), 3.98 (s, CH₂, 2H), 3.65 (m, CH₂, 2H), 3.46 (s, CH₃, 3H), 3.22 (m, CH₂, 4H), 2.77 (d, J = 4.5 Hz, CH₃, 3H), 1.42 (m, CH₂, 4H), 1.23 (m, CH₂, 4H). MS 787.61 (M⁺ + H), 809.65 (M⁺ + Na).

Oxymorphylidiglycolamide (16). A suspension of diglycolic anhydride (0.645 g, 5.56 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise to oxymorphamine **15** (1.6 g, 5.29 mmol) in anhydrous CH₂Cl₂ (10 mL) under N₂. The mixture was stirred at room temperature overnight. The precipitate was collected by suction filtration and washed with CH₂Cl₂ to give 2.20 g (99.4%) of product **16**. ¹H NMR (DMSO-*d*₆): δ 8.75 (m, CONH, 1H), 6.59 (d, J = 7.8 Hz, ArH, 1H), 6.51 (d, J = 7.8 Hz, ArH, 1H), 4.45 (m, 1H), 4.32 (m, 1H), 3.92 (m, 4H), 3.16 (s, 1H), 2.72 (m, 1H), 2.55 (s, 2H), 2.42–2.25 (m, 5H), 1.69–1.61 (m, 1H), 1.49 (m, 1H), 1.33 (m, 2H), 0.88 (m, 1H). MS 417.14 (M⁺ – H).

[N-(*m*-Nitrophenoxy)ethylidiglycolamidehydrazide-*N'*-oxymorphylidiglycolamide]dihydrazide (19a). To a solution of **16** (69.7 mg, 0.167 mmol) in anhydrous DMSO (0.5 mL) were added DCC (37.8 mg, 0.183 mmol), HOBt (24.8 mg, 0.183 mmol), and a solution of **12a** (52 mg, 0.167 mmol) in anhydrous DMSO (0.5 mL) at room temperature. The mixture was stirred for 24 h at room temperature, and the precipitate was removed by filtration. The filtrate was concentrated and the residual was purified by flash chromatography (CH₂Cl₂/MeOH/NH₄OH = 90:9.5:0.5) to give 70 mg (58.8%) of **19a**. ¹H NMR (CDCl₃): δ 7.80 (d, J = 8.1, 1H), 7.70 (m, 1H), 7.41 (t, J = 8.1 Hz, 2H), 7.21 (m, 2H), 6.69 (d, J = 8.1, 1H), 6.52 (d, J = 8.1 Hz, 1H), 4.58 (s, 2H), 4.23–4.10 (m, 8H), 3.75 (m, 2H), 3.10 (d, J = 18.6 Hz, 1H), 2.77 (m, 1H), 2.62–2.51 (m, 6H), 2.38 (m, 1H), 2.34 (s, 3H), 2.22 (m, 2H), 1.77–1.34 (m, 6H), 1.03 (m, 1H). MS 713.84 (M⁺ + H).

1-(*N*-Oxymorphylidiglycolamide)-2-[1-diglycoldiamide-2-(*m*-nitrophenoxy)ethane]ethane (19b). To a solution of **12** (141 mg, 0.473 mmol) and **18b** (198 mg, 0.43 mmol) in anhydrous DMSO (2.5 mL) were added DCC (97.6 mg, 0.473 mmol) and HOBt (64 mg, 0.473 mmol). The reaction solution was stirred overnight at room temperature and concentrated. The residue was purified by a flash chromatography (CH₂Cl₂/CH₃OH/NH₃H₂O = 90:10:1) to give 220 mg (69.1%) of **19b**. ¹H NMR (CDCl₃): δ 7.83 (d, J = 8.1 Hz, 1H), 7.74 (m, 1H), 7.54 (t, J = 6.0 Hz, 1H), 7.44 (m, 1H), 7.35–7.21 (m, 2H), 6.99 (d, J = 8.4 Hz, 1H), 6.70 (d, J = 8.4 Hz, 1H), 6.55 (d, J = 8.4 Hz, 1H), 4.58–4.53 (m, 2H), 4.19 (t, J = 5.7 Hz, 2H), 4.07 (m, 8H), 3.76 (q, J = 5.7 Hz, 2H), 3.61–3.49 (m, 4H), 3.13 (d, J = 18.0 Hz, 1H), 2.80 (m, 1H), 2.69–2.54 (m, 5H), 2.41 (m, 1H), 2.35 (s, 3H), 2.25 (m, 2H), 1.83–1.69 (m, 8H), 1.55 (d, J = 9.6 Hz, 1H), 1.44–1.36 (m, 1H), 1.25 (s, 1H), 1.03–0.88 (m, 1H). MS 741.24 (M⁺ + H).

1-(*N*-Oxymorphylidiglycoldiamide)-6-[1-diglycoldiamide-2-(*m*-nitrophenoxy)ethane]hexane (19c). To an ice-cold solution of **12** (109 mg, 0.366 mmol), DCC (75.4 mg, 0.366 mmol), and HOBt (49.4 mg, 0.366 mmol) in dry DMF (1 mL) was added a solution of **18c** (171.7 mg, 0.332 mmol) in dry DMF (1.5 mL). The reaction mixture was stirred overnight at room temperature. The precipitate was filtered, and the filtrate was concentrated by a rotary-evaporator. The residue was purified by a flash chromatography (CH₂Cl₂/CH₃OH/NH₃H₂O = 90:10:1) to give 255 mg (87.4%) of **19c**. ¹H NMR (CDCl₃): δ 7.84 (d, J = 7.8 Hz, 1H), 7.72 (m, 1H), 7.46 (t, J = 7.8 Hz, 1H), 7.27 (m, 1H), 7.16 (m, 1H), 6.74 (m, 2H), 6.54 (d, J = 7.2 Hz), 4.59 (m, 2H), 4.16 (m, 2H), 4.08 (m, 4H), 4.05 (m, 4H), 3.76 (m, 2H), 3.33 (m, 3H), 2.62 (s, 8H), 2.35 (s, 3H), 2.25 (m, 2H), 1.71 (m, 5H), 1.56 (m, 3H), 1.36 (m, 4H), 1.01 (m, 1H). MS 797.62 (M⁺ + H), 819.60 (M⁺ + Na).

***N*-(*m*-Aminophenoxy)ethylidiglycolamidehydrazide-*N'*-oxymorphylidiglycolamidedihydrazide (20a).** A mixture of **19a** (66 mg, 0.093 mmol) and Pd/C of 10 wt % (15 mg) in MeOH (4 mL) was hydrogenated at 50 psi for 4 h at room temperature. The catalyst was removed by filtration over a Celite pad, and the filtrate was concentrated in a rotary-evaporator to give 60 mg (94.5%) of product **20a**. ¹H NMR (CDCl₃): δ 8.03 (s br, 1H), 7.52 (s br, 1H), 6.99 (dd, J = 7.8 Hz, 1H), 6.69 (d, J = 7.8 Hz, 1H), 6.53 (d, J = 7.8 Hz, 1H), 6.26 (m, 2H), 4.57 (s, 2H), 4.18–4.01 (m, 8H), 3.63 (m, 2H), 3.12 (d, J = 18.3 Hz, 1H), 2.73–2.55 (m, 11H), 2.40 (m, 1H), 2.35 (s, 3H), 2.28–2.18 (m, 2H), 1.81–1.70 (m, 1H), 1.55 (m, 2H), 1.44–1.36 (m, 1H), 1.04 (m, 1H). MS 683.35 (M⁺ + H).

1-(*N*-Oxymorphylidiglycoldiamide)-2-[1-diglycoldiamide-2-(*m*-aminophenoxy)ethane]ethane (20b). A mixture of **19b** (120 mg, 0.162 mmol) and Pd/C of 10 wt % (30 mg) in MeOH (6.5 mL) was hydrogenated at 50 psi for 4 h at room temperature. The catalyst was removed by suction filtration and the filtrate was concentrated by a rotary-evaporator to give 100 mg (86.8%) of product **20b**. ¹H NMR (CDCl₃): δ 7.58 (m, 1H), 7.48 (t, J = 5.7 Hz, 1H), 7.39 (m, 1H), 7.10–7.06 (m, 1H), 7.02 (d, J = 8.1 Hz, 1H), 6.70 (d, J = 8.1 Hz, 1H), 6.54 (d, J = 8.1 Hz, 1H), 6.31–6.23 (m, 3H), 4.59–4.52 (m, 2H), 4.08–3.97 (m, 8H), 3.88 (m, 2H), 3.68 (q, J = 5.4 Hz, 2H), 3.49 (s, 6H), 3.12 (d, J = 18.6 Hz, 1H), 2.78 (d, J = 6.0 Hz, 1H), 2.69–2.53 (m, 10H), 2.40 (m, 1H), 2.35 (s, 3H), 2.28–2.21 (m, 2H), 1.92–1.36 (m, 6H), 1.25 (m, 1H), 1.00 (m, 1H). MS 711.21 (M⁺ + H).

1-(*N*-Oxymorphylidiglycoldiamide)-6-[1-diglycoldiamide-2-(*m*-aminophenoxy)ethane]hexane (20c). A mixture of **19c** (250 mg, 0.314 mmol) and Pd/C of 10 wt % (80 mg) in MeOH (13 mL) was hydrogenated at 50 psi for 4 h at room temperature. The catalyst was removed by filtration over a Celite pad and the filtrate was concentrated in a rotary-evaporator to give 110 mg (45.7%) of product **20c**. ¹H NMR (DMSO-*d*₆): δ 8.21 (m, 1H), 8.03 (m, 2H), 7.57 (m, 1H), 6.86 (t, J = 7.5 Hz, 1H), 6.56 (d, J = 8.1 Hz, 1H), 6.46 (d, J = 8.1 Hz, 1H), 6.11–6.04 (m, 3H), 5.02 (m, 2H), 4.78 (s, 1H), 4.44 (m, 1H), 3.93–3.88 (m, 8H), 3.44 (q, J = 6.0 Hz, 2H), 3.32 (s, 2H), 3.06 (m, 4H), 2.71 (d, J = 6.0 Hz, 1H), 2.52 (m, 6H), 2.36 (m, 1H), 2.27 (s, 3H), 2.13 (m, 2H), 1.56 (m, 1H), 1.40 (m, 5H), 1.23 (m, 3H), 0.92 (m, 1H). MS 767.69 (M⁺ + H), 789.68 (M⁺ + Na).

***N*-[*m*-(*R*(+)-1)Ureaphenoxy]ethylidiglycolamidehydrazide-*N'*-oxymorphylidiglycolamidedihydrazide (3a).** To a solution of **20a** (57 mg, 0.0835 mmol) in anhydrous DMSO (0.5 mL) was added a solution of **8** (26.8 mg, 0.0916 mmol) in anhydrous DMSO (0.5 mL). The reaction solution was stirred at room temperature for 24 h and concentrated. The residue was purified by flash chromatography (CH₂Cl₂/MeOH/NH₄OH = 85/15/1.5) to give 10 mg (12.3%) of product **3a**. ¹H NMR (CDCl₃): δ 7.65–7.33 (m, 10H), 7.30–7.22 (m, 2H), 6.97 (dd, J = 7.8 Hz, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.66 (d, J = 7.8 Hz, 1H), 6.31–6.23 (m, 2H), 5.29 (d, J = 6.0 Hz, 1H), 4.58 (m, 2H), 4.17–3.96 (m, 8H), 3.91–3.75 (m, 3H), 3.61 (m, 3H), 3.49 (s, 3H), 3.16 (d, J = 18.9 Hz, 1H), 2.81 (m, 1H), 2.41 (s, 3H), 2.22 (m, 2H), 1.97–1.67 (m, 4H), 1.59–1.48 (m, 2H), 1.41–1.38 (m, 2H), 1.00 (m, 1H). MS 974.36 (M⁺ + H).

1-{1-Diglycoldiamide-2-[*m*-(*R*(+)-1)ureaphenoxy]ethane}-2-(*N*-oxymorphylidiglycoldiamide)ethane (3b). To a solution of **20b** (95.7 mg, 0.135 mmol) in anhydrous DMSO (2 mL) was added a solution of **8** (39.2 mg, 0.135 mmol) in anhydrous THF (0.5 mL). The reaction solution was stirred at room temperature for 24 h and concentrated. The residue was purified by flash chromatography (CH₂Cl₂/MeOH/NH₄OH = 90/10/1) to give 24.9 mg (18.4%) of product **3b**. ¹H NMR (CDCl₃): δ 8.10 (s, 1H), 7.61–7.52 (m, 3H), 7.49–7.42 (m, 2H), 7.39–7.32 (m, 2H), 7.26–7.15 (m, 4H), 6.98 (d, J = 9.0 Hz, 1H), 6.92 (m, 1H), 6.71–6.61 (m, 2H), 6.53–6.50 (m, 2H), 5.50 (d, J = 7.8 Hz, 1H), 5.30 (s, 2H), 4.74–4.64 (m, 2H), 4.54–4.49 (m, 2H), 4.10–4.03 (m, 8H), 3.96 (m, 2H), 3.67 (m, 2H), 3.46 (m, 3H), 3.44 (s, 3H), 3.15–3.08 (m, 2H), 2.84–2.76 (m, 2H), 2.65–2.51 (m, 2H), 2.46–2.18 (m, 4H), 2.34 (s, 3H), 1.88–1.36 (m, 9H), 1.25 (s, 1H), 1.02–0.86 (m, 2H). MS 1002.20 (M⁺ + H).

1-{1-Diglycoldiamide-6-[*m*-(*R*(+)-1)ureaphenoxy]ethane}-2-(*N*-oxymorphylidiglycoldiamide)hexane (3c). To a solution of **20c** (194.8 mg, 0.254 mmol) and **8** (120 mg, 0.279 mmol) in anhydrous DMSO (5 mL) was added triethylamine (50 μ L, 0.279 mmol). The reaction solution was stirred at room temperature for 6 h and concentrated. The residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH} = 90/10/1$) to give 21.1 mg (7.9%) of product **3c**. ^1H NMR (CDCl_3): δ 7.65–7.58 (m, 2H), 7.50–7.32 (m, 6H), 7.24–7.13 (m, 3H), 7.02 (t, $J = 7.8$ Hz, 1H), 6.92 (d, $J = 8.1$ Hz, 1H), 6.81–6.72 (m, 2H), 6.67 (d, $J = 8.1$ Hz, 1H), 6.29–6.20 (m, 2H), 5.30 (d, $J = 7.8$ Hz, 1H), 4.58 (d, $J = 4.2$ Hz, 1H), 4.43 (m, 1H), 4.06–3.99 (m, 8H), 3.72–3.55 (m, 3H), 3.49 (s, 3H), 3.25–3.08 (m, 4H), 2.81 (m, 1H), 2.62 (s, 4H), 2.35 (s, 3H), 2.24 (m, 2H), 1.88–1.56 (m, 8H), 1.41 (m, 6H), 1.25 (m, 6H), 0.95 (m, 1H). MS 1058.44 ($\text{M}^+ + \text{H}$), 1080.42 ($\text{M}^+ + \text{Na}$).

***N*-(*m*-Nitrophenoxy)ethyl-*N'*-oxymorphylidiglycoldiamide (22).** To a solution of **16** (209 mg, 0.5 mmol) in anhydrous DMSO (1.5 mL) were added DCC (113 mg, 0.55 mmol) and HOBt (74.3 mg, 0.55 mmol), and the solution was stirred for 5 min. A solution of 1-amino-2-(3-nitrophenoxy)ethane **21** (100 mg, 0.55 mmol) in anhydrous DMSO (1.0 mL) was added to the reaction solution, and the reaction mixture continued stirring overnight at room temperature. The precipitate was removed by filtration and the residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH} = 92:7.5:0.5$) to give product **22** as oil (223.7 mg). ^1H NMR (CDCl_3): δ 7.84–7.80 (m, 1H), 7.70 (m, 1H), 7.42 (t, $J = 8.4$ Hz, 1H), 7.22–7.19 (m, 2H), 6.97 (m, 1H), 6.71 (d, $J = 8.4$ Hz, 1H), 6.56 (d, $J = 8.4$ Hz, 1H), 4.60–4.55 (m, 2H), 4.18–4.14 (m, 4H), 4.07 (d, $J = 6.6$ Hz, 1H), 3.84–3.77 (m, 2H), 3.12 (m, 1H), 2.84–2.68 (m, 2H), 2.40 (m, 1H), 2.34 (s, 3H), 2.27–2.21 (m, 1H), 1.79–1.52 (m, 6H), 1.37–1.25 (m, 2H), 0.92 (m, 1H). MS 583.14 ($\text{M}^+ + \text{H}$).

***N*-(*m*-Aminophenoxy)ethyl-*N'*-oxymorphylidiglycoldiamide (23).** A mixture of **22** (220 mg, 0.338 mmol) and Pd/C (10 wt %, 72 mg) in MeOH (13 mL) was hydrogenated at room temperature and 50 psi for 4 h. The catalyst was removed by filtration over a Celite pad and the filtrate was concentrated in a rotary-evaporator to give 196 mg of product **23**. ^1H NMR ($\text{DMSO}-d_6$): δ 8.25 (s br, 1H), 7.57 (m, 1H), 6.85 (t, $J = 8.4$ Hz, 1H), 6.56 (d, $J = 8.4$ Hz, 1H), 6.45 (d, $J = 8.4$ Hz, 1H), 6.12–6.03 (m, 3H), 5.01 (s, 2H), 4.78 (s, 1H), 4.44 (m, 1H), 4.37–4.30 (m, 1H), 3.98 (m, 4H), 3.87 (t, $J = 6.0$ Hz, 2H), 3.43 (q, $J = 6.0$ Hz, 2H), 3.02 (d, $J = 18.6$ Hz, 1H), 2.53 (m, 3H), 2.37–2.32 (m, 1H), 2.27 (m, 3H), 2.17–2.10 (m, 2H), 1.58–1.51 (m, 1H), 1.33 (m, 3H), 0.92 (m, 1H). MS 553.20 ($\text{M}^+ + \text{H}$).

***N*-(*m*-[*R*(+)-1]Ureaphenoxy)ethyl-*N'*-oxymorphylidiglycoldiamide (4).** To a solution of **23** (193 mg, 0.349 mmol) in anhydrous DMSO (4.5 mL) was added a solution of **8** (96.6 mg, 0.332 mmol) in anhydrous DMSO (2 mL). The reaction solution was stirred at room temperature for 24 h and concentrated. The residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH} = 90:9:1$) to give 21 mg (7.5%) of **4** as a solid. ^1H NMR (CDCl_3): δ 7.60 (m, 4H), 7.47–7.20 (m, 8H), 7.00 (m, 2H), 6.75 (m, 1H), 6.65 (d, $J = 8.1$ Hz, 1H), 6.23 (d, $J = 8.1$ Hz, 1H), 6.13 (m, 2H), 5.34 (d, $J = 8.1$ Hz, 1H), 4.56 (m, 1H), 4.39 (m, 1H), 4.03 (m, 1H), 3.88 (t, $J = 5.4$ Hz, 1H), 3.77–3.63 (m, 2H), 3.58 (s, 1H), 3.48 (m, 3H), 3.44–3.39 (m, 2H), 3.14 (d, $J = 18.9$ Hz, 1H), 2.78 (m, 1H), 2.67–2.58 (m, 1H), 2.41 (m, 1H), 2.34 (s, 3H), 2.25–2.17 (m, 2H), 1.71–1.50 (m, 6H), 1.38–1.20 (m, 3H), 0.89 (m, 1H). MS 844.34 ($\text{M}^+ + \text{H}$).

Biology. 1. In Vitro Studies. 1.1. Preparation of Receptor Constructs. Type 2 cholecystokinin (CCK_2) and MOP receptor constructs tagged at their carboxyl-terminal ends with either *Renilla* luciferase (Rlu) or yellow fluorescent protein (YFP) were used as donor and acceptor, respectively, for BRET studies. We previously characterized the Rlu- and YFP-tagged CCK_2 receptor constructs expressed in COS cells, demonstrating that their binding characteristics and biological activities are similar to those of wild type CCK_2 receptor.³⁴ The Rlu- and YFP-tagged MOP receptor constructs were prepared utilizing the Rlu and YFP sequences present in pBRET and pEYFP-N1 (Stratagene, La Jolla, CA) and cloning

these in frame at the 3'-end of the receptor sequence into NheI/XbaI sites of pcDNA3.0 vector (Invitrogen, Carlsbad, CA). All sequences were verified by direct DNA sequencing.

1.2. Receptor-Bearing Cells. Chinese hamster ovary (CHO-K1) cells stably expressing the CCK_2 receptors, the MOP receptors, and cells coexpressing both of these receptors were prepared and used as sources of receptor-enriched plasma membranes for receptor binding studies. CHO cells obtained from the American Type Culture Collection (Manassas, VA) and receptor-expressing CHO cell lines were grown in Ham's F-12 nutrient mixture containing 5% (v/v) Fetal Clone II supplement (Hyclone laboratories, Logan, UT) in a 5% CO_2 environment. Cells were passaged approximately twice per week. Cell lines stably expressing receptor constructs were prepared by transfection with appropriate cDNA. Cells were selected with 1 mg/mL G-418 or 0.5 mg/mL zeocin and then were subjected to a series of standard limiting dilutions, followed by screening using radioligand binding to assess receptor expression.

COS cells transiently expressing receptor constructs were used for receptor BRET studies. These cells were plated in 10 cm tissue culture dishes at a density of 0.5 million cells/dish and were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Fetal Clone II. Cells were transfected 48 h before use with approximately 3 μ g of total DNA (either a single construct or a combination of two constructs (donor and acceptor)) per dish using the DEAE-dextran method.⁴²

1.3. CCK Receptor Binding. Receptor-enriched membranes were isolated from CHO cell lines using the previously established density gradient centrifugation procedure.⁴³ The membranes were suspended in Krebs-Ringers-HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM KH_2PO_4 , 1.2 mM MgSO_4) containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride and were stored at -80°C until use. Radioligand binding studies were performed as described previously.⁴⁴ Receptor preparations were mixed with a constant amount of CCK-like radioligand, [^{125}I]-D-Tyr-Gly-[Nle28,31]CCK-26-33 (1–5 pM) (specific radioactivity 2000 Ci/mmol), in the absence or presence of increasing concentrations of unlabeled ligand for 90 min at room temperature in KRH medium, pH 7.4, containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. Receptor-bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with receptor-binding filtermats. Receptor-bound radioactivity was quantified using a γ spectrometer. Nonsaturable binding determined in the presence of 1 μM CCK was consistently less than 10% of total binding. Saturable radioligand binding data were analyzed using the LIGAND program⁴⁵ and were plotted using the nonlinear least-squares curve-fitting routine in Prism (GraphPad 3.0, San Diego, CA).

1.4. MOP Receptor Binding. Opioid receptor binding was performed with membranes isolated from CHO cells (Table 1). Receptor-enriched membranes were mixed with a constant amount of specific MOP radioligand [^3H]DAMGO (0.4 nM) (specific radioactivity 56.8 Ci/mmol) (Perkin-Elmer, Boston, MA) in the absence or presence of increasing concentrations of unlabeled ligand for 2 h at room temperature in KRH medium, pH 7.4, containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. The nonspecific binding was determined in the presence of 1 μM DAMGO (Bachem, Torrance, CA), an agonist ligand for the MOP receptor. Receptor-bound and free radioligand were separated by rapid filtration using Whatman GF/C glass microfiber filters (Whatman Inc., Florham Park, NJ) that were presoaked in 0.3% polyethylenimine (Sigma, St. Louis, MO). Receptor-bound radioactivity was quantified in a Beckman-Coulter LS6500 multipurpose scintillation counter. Saturable radioligand binding data were analyzed using the LIGAND program⁴⁵ and were plotted using the nonlinear least-squares curve-fitting routine in Prism (GraphPad 3.0, San Diego, CA).

Values are expressed as mean values \pm SEM of data from three to five independent experiments.

1.5. BRET Studies. Bioluminescence and fluorescence measurements were performed with aliquots of approximately 25 000 receptor-bearing COS cells in suspension, as described previously.⁴⁴ For this, the cells were lifted using nonenzymatic cell dissociation solution (Sigma) 48 h after transient transfection, and they were washed with KRH medium. The BRET assay was initiated by adding the cell-permeant *Renilla* luciferase-specific substrate, coelenterazine *h* (Biotium, Hayward, CA), to the cell suspension to yield a final concentration of 5 μ M in a 96-well white Optiplate (PerkinElmer, Boston, MA). The BRET signal was collected by using the protocol designed for BRET studies in the 2103 Envision fluorescence plate reader (Perkin-Elmer, Boston, MA), and the BRET ratio was calculated based on emission ratios. For ligand effects, the transfected COS cells were incubated with specified ligands (1 μ M) for 90 min at room temperature before performing the assay. Saturation BRET experiments were performed as described previously.⁴⁴ COS cells were transiently transfected with a fixed amount of Rlu-tagged μ -opioid receptor construct (1.0 μ g DNA/dish) and with increasing concentrations of YFP-tagged CCK₂ receptor construct (0.3–6 μ g DNA/dish). Curves were fit to these data and evaluated for quality-of-fit based on R^2 values using Prism 3.0, and the BRET₅₀ and BRET_{max} values were calculated.

1.6. Calcium Mobilization.³⁶ Calcium mobilization was done in MOPR-CCK₂R coexpressing CHO cells. Stably expressing CHO CCK₂R cells were grown to 90% confluent in HAM F-12 nutrient media plus 10% bovine calf serum and 1% penicillin/streptomycin in 75 mm² tissue culture plates at 37 °C and were transiently transfected with MOPR construct and chimeric G-protein (G_{q14-myr}) construct using lipofectamine 2000 transfection reagent and manufacturer's protocol (GIBCO). Ratios were 20 μ g of MOPR construct, 24 μ g of G_{q14-myr} construct to 80 mL of lipofectamine reagent. Cells were incubated for 24 h after transfection, seeded into 96-well plates (Corning 96-well plates half area, black with clear bottom), and incubated overnight before experiments were conducted. Calcium mobilization was measured on a FlexStation3 using a FLIPR calcium kit (Molecular Devices) and analyzed using SoftMax Pro and PRISM software.

2. In Vivo Studies. 2.1. Materials and Methods. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Minneapolis, MN).

2.2. Subjects. Male ICR mice (17–25 g; Harlan, Madison, WI) were housed in groups of 8 in a temperature- and humidity-controlled environment with unlimited access to food and water. They were maintained on a 12-h light/dark cycle.

2.3. Tail Flick Assay. The tail flick assay was used to test for antinociception described by D'Amour and Smith⁴⁶ and modified by Dewey et al.⁴⁷ 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, Columbus, Ohio) 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 Pierson⁴⁸ as the percent maximal possible effect (% MPE) which is calculated as

$$\% \text{ MPE} = \frac{\text{test} - \text{control}}{10 - \text{control}} \times 100$$

At least three groups of eight to ten mice were used for each dose response curve, and each mouse was used only once. ED₅₀ values with 95% confidence intervals (CI) were computed with GraphPad Prism 4 by using nonlinear regression methods.

2.4. Experimental Design. All the compounds were dissolved in 10% DMSO and then diluted to less than 1% DMSO in the test solutions. Controls given icv with 1% or less DMSO did not show any antinociception. All drugs were administered in a 5 μ L volume

in conscious mice according to the method of Haley and McCormick⁴⁹ for icv injections. A time course study, including the times 10, 20, 30, and 60 min was used to determine the peak antinociception. List of compounds and peak times are summarized in the Table 3. A 24 h tolerance was measured with each compound using the ED_{80–90} doses measured on day 1 and then repeated 24 h later on the same mouse.

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