Trafficking of Preassembled Opioid $\mu-\delta$ Heterooligomer−Gz Signaling Complexes to the Plasma Membrane: Coregulation by Agonists[†]

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Received July 20, 2007; Revised Manuscript Received September 7, 2007

ABSTRACT: The cellular site of formation, Gα-coupling preference, and agonist regulation of $\mu-\delta$ opioid receptor (OR) heterooligomers were studied. Bioluminescence resonance energy transfer (BRET) showed that $\mu-\delta$OR heterooligomers, composed of preformed μ and δ homooligomers, interacted constitutively in the endoplasmic reticulum (ER) with Gα-proteins forming heteromeric signaling complexes before being targeted to the plasma membrane. Compared to μOR homooligomers, the $\mu-\delta$ heterooligomers showed higher affinity and efficiency of interaction for Gz over Gi, indicating a switch in G-protein preference. Treatment with DAMGO or deltorphin II led to coregulated internalization of both receptors, whereas DPDPE and DSLET had no effect on $\mu-\delta$ internalization. Staggered expression resulted in noninteracting μ and δ receptors, even though both receptors were colocalized at the cell surface. Agonists failed to induce BRET between staggered receptors, and resulted in internalization solely of the receptor targeted by agonist. Thus, $\mu-\delta$OR heterooligomers form and preferentially associate with Gz to generate a signaling complex in the ER, and have a distinct agonist-internalization profile compared to either μ or δ homooligomers.

Three genes encode the known opioid G protein coupled receptors (GPCRs[1]) μ, δ, and κ (*1*, *2*) which share 60 to 65% homology (*3*). Multiple pharmacological subtypes of these receptors have been described, based on ligand binding and activation profiles in native tissues (*4*). One plausible mechanism by which the generation of receptor subtypes could occur may be through oligomerization of these receptors to each other and to other GPCRs, generating diverse signaling units (*3*, *5*). For GPCR superfamily members, recognition of the significance of oligomerization is rapidly advancing and changing our understanding of GPCR regulation and function (*5*−*7*).

We and others were among the first to supply biochemical and pharmacological evidence for GPCR homo- and heterooligomerization (*8*−*13*). For opioid receptors, the formation of $\mu-\delta$ receptor complexes with novel pharmacological and signaling properties was identified through co-immunoprecipitation, ligand binding, and resonance energy transfer experiments (*8*, *9*, *14*, *15*). The existence of $\kappa-\delta$ (*8*, *16*, *17*), and $\mu-\kappa$ opioid receptor heterooligomers (*17*) has also been documented. The evidence for the formation of novel complexes has been strengthened by pharmacological studies showing different ligand binding profiles for heterooligomers (*8*, *9*). Novel functional properties for these complexes have also been described (*3*), including our observation that agonist activation of $\mu-\delta$ complexes showed no sensitivity to pertussis toxin (PTX), in contrast with μ and δ homooligomers in the transduction of their signal (*9*, *18*). The physiological relevance of the $\mu-\delta$ interactions is highlighted in receptor knockout mice, since studies on μ-deficient mice showed that δ-mediated nociception and respiratory depression were reduced or abolished, suggesting a functional interaction between μ and δ receptors *in vivo* (*19*, *20*).

Evidence for homooligomerization to occur before the receptors traffic to the plasma membrane has been documented (*21*, *22*), including for μ, κ, and δ opioid homooligomers (*17*). However, many questions regarding the function, regulation, and biosynthesis of the heterooligomeric complexes remain poorly understood or controversial. We here present data showing that μ and δ receptors interacted constitutively at an early stage of their processing in endoplasmic reticulum (ER), forming heterooligomers from preexisting homooligomers. This μ and δ receptor interaction occurred only when the receptors were expressed concurrently in the ER, and not when their expression was staggered. Furthermore, the formation of $\mu-\delta$ heterooligo-

[†] The work was supported by the U.S. National Institute on Drug Abuse and the Canadian Institutes for Health Research.

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[1] Abbreviations: GPCR, G-protein coupled receptor; BRET, bioluminescence resonance energy transfer; μOR, mu opioid receptor; δOR, delta opioid receptor; ER, endoplasmic reticulum; Rluc, *renilla* luciferase; mRFP, mono red fluorescent protein; GFP, green fluorescent protein; DAMGO, [Tyr-D-Ala[2]-Gly-*N*-methyl-Phe[4],Gly[5]-ol]enkephalin; DEL II, deltorphin II; DPDPE, [D-Pen[2],D-Pen[5]]enkephalin; DSLET, [D-Ser[2],Leu[5]]enkephalin-Thr; PTX, pertussis toxin.

10.1021/bi701436w CCC: $37.00
Published on Web 10/17/2007

meric complexes led to a switch in the preference of μOR for Gz over Gi, based on whether the receptor was in a hetero- or homooligomeric arrangement. The interaction with G proteins was detected not only at the cell surface but also in the ER, suggesting that the receptor−G protein interaction and signaling complex formation occurred before trafficking to the cell surface. We also report that exposure to selective agonists induced cointernalization of the $\mu-\delta$ heterooligomeric complex as a unit, distinct from the non-interacting μ and δ receptors.

MATERIALS AND METHODS

Cell Culture. Human embryonic kidney (HEK)-293 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Burlington, Canada) supplemented with 4% foetal bovine serum (FBS; GIBCO), and 1% antibiotic−antimycotic solution (penicillin, 1000 U/mL; streptomycin, 10 mg/mL; GIBCO). The cells were allowed to grow at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. At 90% confluence, they were dissociated using a pasteur pipet.

cDNA Constructs. cDNAs encoding the rat μ- and δ-ORs were inserted separately into the mammalian expression vector pcDNA3. For immunoprecipitation and radioligand binding studies, μ and δ were tagged at their N-termini with c-Myc and FLAG epitopes respectively, as previously described (*9*). For BRET studies, μ and δ were tagged at their C-termini with *Renilla* luciferase (Rluc, Invitrogen) and Green Fluorescent Protein (GFP, Invitrogen), respectively. For live-cell confocal microscopy, μ and δ were tagged at their C-termini with the monomerized Red Fluorescent Protein (mRFP, Invitrogen) and GFP, respectively. The correct orientations of the polymerase chain reaction (PCR) products in the expression vectors were verified by sequencing on all strands.

Cell Transfection. All transfections were performed using the Effectene reagent kit (QIAGEN, Valencia, CA). HEK-293 cells were transiently transfected with the μ-Rluc cDNA either alone or together with δ-GFP cDNA for BRET studies. For immunoprecipitation and radioligand binding studies, cells were transfected with Myc-μ cDNA along with FLAG-δ cDNA. For confocal microscopy, cells were transfected with μ-mRFP cDNA alone or with δ-GFP cDNA in 60 mm dishes. A total amount of no more than 2 μg/100 mm dish or 1 μg/60 mm dish of DNA was used for the experiments. Twenty-four hours post-transfection, cells were transferred to 6-, 24-, or 96-well plates, and experiments were performed 48 h post-transfection.

Cell Treatment and Solubilization. HEK-293 cells were treated, when indicated, with 100 nM deltorphin II (Del II) or [D-Ala^2,*N*-methyl-Phe^4,Gly^5-ol]enkephalin (DAMGO) for different times. Cells were then washed twice with phosphate buffered saline (PBS, pH 7.4) and lysed for 30 min at 4 °C using cold lysis buffer [25 mM HEPES (pH 7.4), 5 mM EDTA, 50 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate], supplemented with a mixture of protease inhibitors (1 μg/mL pepstatin A, 10 μg/mL leupeptin, 5 μg/mL aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). The solubilized cells were cleared by centrifugation at 21000*g* for 35 min at 4 °C, and the supernatant was recovered for immunoprecipitation or immunoblotting experiments. The protein content was measured using a Bradford reagent kit (Bio-RAD) and BSA as standard.

Radioligand Binding Studies. Radioligand binding studies were performed on intact attached HEK-293 cells expressing μOR and/or δOR as previously described (*23*−*25*) with minor modifications. Twenty-four hours post-transfection, the cells were seeded in 24-well plates, and 48 h post-transfection, radioligand binding studies were performed. Cells were pretreated with 100 nM deltorphin II or DAMGO for 1 h at 37 °C. Ligand binding was performed in 200 μL of 50 mM Tris-HCl with final concentrations of 2.5 nM [^{3}H]-DAMGO, [^{3}H]naloxone, or [^{3}H]diprenorphine in the presence (nonspecific binding) or the absence (total binding) of 10 μM naltrexone or naloxone, and incubated at 4 °C for 4.5 h. Cells were washed twice with 50 mM Tris-HCl, and 250 μL of 0.2 N NaOH was added. Cells were then kept at 4 °C for 30 min before being harvested. Radioactivity was then determined by counting, and the results were analyzed using Prism (Graph Pad).

Bioluminescence Resonance Energy Transfer (BRET) Assay. To detect and analyze interactions between μ and δ receptors, BRET studies were performed. For this purpose, cells were transfected with μ-Rluc with or without cotransfection with plasmids encoding δ receptor, muscarinic M4 receptor, Gαz or Gαi proteins, tagged with GFP (δ-GFP, M4-GFP, Gz-GFP, Gi-GFP). The Gi-GFP cDNA construct was prepared as described (*26*), and Gz-GFP was prepared in a similar manner by inserting the EGFP between positions 91 and 92 in the Gα proteins. The insertions into this position do not affect the activities of G proteins as has been shown (*26*, *27*). Cells were seeded into 96-well plates at a density of 10^5 cells/well. After the induction of Rluc-mediated light emission by the addition of the substrate, Coelenterazine h (Molecular Probes, Eugene, OR), emission was measured using a plate-reader spectrofluorometer (Victor^3, Perkin-Elmer) at the wavelengths of 480 and 535 nm, corresponding to the maxima of the emission spectra for Rluc and GFP, respectively. The BRET ratio was calculated using the equation previously described (*28*).

Immunoprecipitation and Immunoblotting. For co-immunoprecipitation experiments, cells expressing Myc-μOR alone or with FLAG-δOR were washed twice with cold PBS and solubilized as indicated above. An anti-Myc mouse monoclonal antibody (SantaCruz) was used to immunoprecipitate the Myc-μOR from cell lysates (at 4 °C overnight). ProteinG-agarose (Pierce) was then added for 1 h. Beads were washed three times with cold lysis buffer, and SDS−PAGE buffer was added to each sample. Proteins were resolved by electrophoresis on polyacrylamide gels under denaturing conditions (SDS−PAGE) and transferred onto nitrocellulose or PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using a semidry transfer system (Invitrogen). Membranes were incubated in PBS-T/10% nonfat milk for 1 h. After three washes in PBS, membranes were incubated with PBS-T/10% nonfat milk containing the indicated first antibody (anti-FLAG or anti-Myc at 4^{o}C overnight). Membranes were washed 3 times for 5 min in PBS-T and incubated with a horseradish peroxidase (HRP)-conjugated polyclonal secondary antibody (Bio-Rad) for 2 h. After washes as indicated above, detection was performed using a chemiluminescence kit (Perkin-Elmer).

Cell Fractionation by Sucrose Gradient . Forty-eight hours post-transfection with cDNA, cells were washed with cold PBS, scraped off, and lysed on ice for 30 min with cold lysis buffer (indicated above). The supernatant was cleared by centrifugation at 21000*g* for 35 min at 4 °C and supplemented with sucrose to achieve a final concentration of 0.2 M sucrose. A discontinuous sucrose step gradient (10−60%) was made using cold lysis buffer. Cell lysate was applied to the top of the gradient, and samples were centrifuged for 19 h at 110000*g* at 4 °C. Aliquots (30 μL) from each fraction were used to identify the plasma membrane and endoplasmic reticulum enriched fractions by Western blot, using antibodies raised against Na^+/K^+-ATPase (Sigma, St. Louis, MO) and BiP_{GRP78} (StressGen Biotechnologies Corp., Victoria, BC, Canada), respectively. Fractions were then subjected to fluorescence/luminescence analysis and to BRET analysis as described.

Staggered Receptor Expression. HEK 293 cells were transiently transfected with one receptor 24 h before the other receptor, and experiments were performed 24−48 h later. The total amount of DNA transfected did not exceed 1 to 2 μg, to yield receptor expression densities of 100−150 fmol/mg protein.

Live Cell Confocal Microscopy. For the live cell monitoring of μ- or/and δ-OR, HEK-293 cells in 60 cm dishes were cotransfected with cDNA encoding for μ-mRFP and δ-GFP. Live cell confocal images were acquired 48 h later using an X63 deep lens mounted on a Zeiss LSM510 confocal microscope. The microscope was equipped with a heated stage unit maintained at 37 °C during the experiments. The GFP was excited at 488 nm, with a fluorescence emission captured at 525 nm, whereas the mRFP was excited at 543 nm, with emission captured at 573 nm. The images were acquired using the LSM510 software at 512 × 512 bit resolution in sequential mode to avoid any bleed through.

Immunocytochemistry. Floating paraformaldehyde-fixed sections (10 μm) from rat brain were permeabilized for 2 h with 0.1% Triton X-100 and then incubated for 24 h at 4^{o}C with primary antibodies directed against μ- and δ-ORs (guinea pig anti-μOR, Neuromics, Inc. 1:400; rabbit anti-δOR, 1:1000, Chemicon Intl. Inc.). Sections were then incubated for 2 h at room temperature with secondary antibodies (FITC-conjugated anti-guinea pig IgG and TRITC-conjugated anti-rabbit IgG), mounted onto slides, and visualized using a Zeiss LSM510 confocal microscope. FITC was excited at 488 nm whereas the TRITC was excited at 546 nm, and the emissions were acquired at 520 and 573 nm respectively.

RESULTS

1. Subcellular Localization of μOR and δOR Interactions. In preliminary experiments, HEK-293 cells were transfected with μ-Rluc alone, or cotransfected with δ-GFP at a DNA ratio of 1, with both receptors expressed at 180−200 fmol/mg protein. The BRET ratio from cells coexpressing μOR and δOR indicated that the receptors existed in living cells in close proximity, less than 100 Å. We then investigated further the $\mu-\delta$ interaction. For these experiments, constant levels of 100−150 fmol/mg protein of μ-Rluc were expressed and the DNA ratio of GFP/Rluc was incremented from 0 to 3, by varying the δ-GFP expression level, and monitored by measuring GFP fluorescence (Supporting Information Figure 1). Data obtained from such experiments (Figure 1A) showed a saturation profile suggesting that the donor (μ-Rluc) interacted with the acceptor (δ-GFP) at a low DNA ratio, with $BRET_{max}$ obtained at a DNA ratio of 0.5, and an apparent $BRET_{50}$ obtained at a ratio of 0.05. When μ-Rluc was coexpressed with the M4 muscarinic receptor (M4-GFP) in similar conditions as monitored by measuring Rluc luminescence and GFP fluorescence (Supporting Information Figure 1), a linear and nonspecific BRET signal was detected (Figure 1A), suggesting that the $\mu-\delta$ interaction was specific and not the result of crowding or arbitrary collisions. This indicated close proximity resulting in a specific and strong interaction between the $\mu-\delta$ receptors.

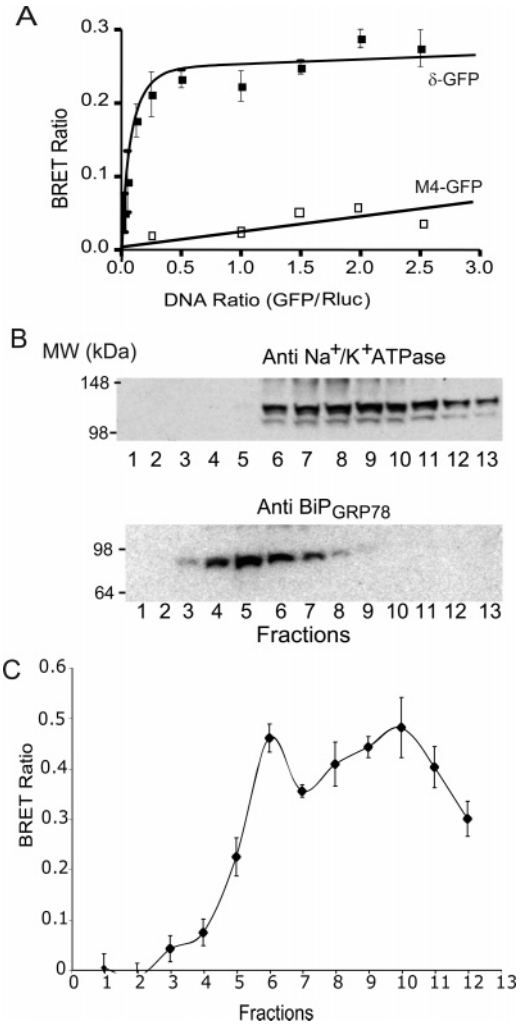

FIGURE 1: Subcellular localization of μOR and δOR interaction. A. A BRET-analysis saturation-curve of $\mu-\delta$ or $\mu-$M4 interaction was performed in cells expressing a constant amount of μ-Rluc (150 fmol/mg protein) alone or with an increasing amount of δ-GFP or M4-GFP. Values shown are the means ± SEM of three independent experiments, each representing 6 measurements. B. HEK-293 cells expressing μ-Rluc and δ-GFP were fractionated on a sucrose gradient. The resulting fractions were separated on SDS−PAGE. Immunoblot of fractions using antibodies to Na^+/K^+ ATPase (plasma membrane marker, upper blot), and BiP_{GRP78} (endoplasmic reticulum marker, lower blot). C. BRET assays performed on each fraction indicate that the receptors interacted with each other to form heterooligomers in the endoplasmic reticulum (fractions 3−7) and on the plasma membrane (fractions 6−13). Values are means ± SEM of three independent experiments, each representing at least 4 measurements.

We next investigated whether $\mu-\delta$OR interactions occurred only when the receptors reach the plasma membrane, or whether it occurred earlier in the receptor biosynthetic pathway. HEK-293 cells transfected with μ-Rluc alone or with δ-GFP were subjected to subcellular fractionation on a sucrose density gradient. An aliquot of each fraction was analyzed by SDS−polyacrylamide gel electrophoresis, followed by immunoblotting for the receptors together with markers for the subcellular fractions. The ER resident chaperone protein BiP_{GRP78} (~78 kDa) was located in fractions 3 to 8, corresponding to the fractions containing exclusively ER membranes (fractions 3−5), while Na^+/K^+ ATPase (~120 kDa) was located in fractions 6 to 12 indicative of the plasma membrane containing fractions (9−12), with fractions (6−8) containing both the ER and the plasma membrane (Figure 1B).

An aliquot of each fraction was subject to BRET analysis. The BRET ratios for the fractions corresponding to the "pure" ER (fractions 3−5) were significant and ranged from 0.05 to 0.25 (Figure 1C). This showed clearly that BRET between the receptors was present in the ER fractions, indicating that μ and δ receptors interacted at an early stage of processing in the ER. For the fractions corresponding to the plasma membrane, the BRET ratios were consistently high, ranging from 0.3 to 0.4, reflecting that μ and δ receptor interactions were maintained at the plasma membrane (Figure 1C). These results suggested that μOR and δOR interacted to form heterooligomers in the ER while being processed and remained heterooligomers at the plasma membrane.

2. Nature of μOR and δOR Species Forming Heterooligomers. In order to analyze the nature of μOR and δOR associated within complexes, co-immunoprecipitation studies were performed. Myc-μOR was immunoprecipitated from HEK-293 cells expressing Myc-μOR alone or with Flag-δOR, followed by immunoblotting the same blot with anti-Flag and then with anti-c-Myc antibodies (Figure 2A). A single δOR band with a molecular weight greater than 250 kDa was immunoprecipitated from cells coexpressing μOR and δOR (Figure 2A, left panel), and not from cells expressing μOR alone, indicating that Flag-δOR co-immunoprecipitated with Myc-μOR in a single oligomeric form. The anti-c-Myc antibody (Figure 2A, right panel) precipitated three different species of μOR from cells expressing μOR alone or coexpressing μOR and δOR, corresponding to monomer (~48−49 kDa), dimer (~98 kDa), and tetramer (~195 kDa). Since only a single δ receptor species was immunoprecipitated using the μ receptor and since the anti-c-Myc and anti-Flag antibodies did not pick up the same higher MW species, this indicated that the >250 kDa species was composed exclusively of δ receptors and the ~195 kDa species exclusively of μ receptors. The size analysis by Western-blot showed that δOR was detected as monomer (65−68 kDa), dimer (130−140 kDa), and a probable tetramer (260−280 kDa) (Supporting Information Figure 2A, right panel). However, only the high molecular weight band of δOR was co-immuprecipitated with μOR, suggesting that μOR interacted with a homooligomer of δOR, probably a homotetramer. These results were neither due to the presence of a particular tag nor to a nonspecific protein aggregation, since the co-immunoprecipitation of Myc-μOR with δOR-GFP resulted in the precipitation of only a single high MW (>250 kDa) δOR species (Supporting Information

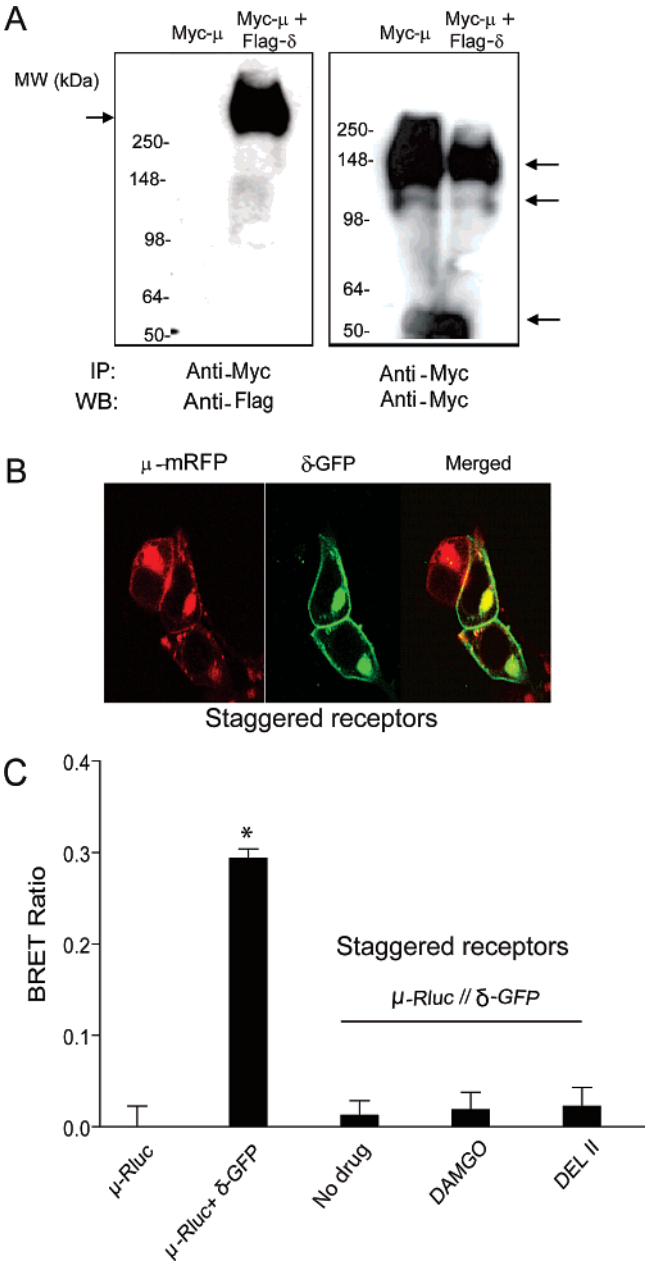

FIGURE 2: Nature of μOR and δOR species involved in heterooligomer formation. Anti-c-Myc antibody was used to immunoprecipitate the Myc-tagged μOR (Myc-μOR) from cells expressing it alone or with FLAG-δOR. A. Immunoblotting with the anti-Flag antibody revealed no immunoreactive bands from cells expressing only Myc-μOR (left panel, lane 1), whereas cells expressing both receptors showed a single band of molecular weight greater than 250 kDa, indicated by the arrow (left panel, lane 2). The same blot was then reprobed with the anti-Myc antibody. Identical bands were observed from cells expressing Myc-μOR alone (right panel, lane 1) and in cells expressing both receptors (right panel, lane 2). The arrows indicate the molecular weights of Myc-μOR monomer, dimer, and higher oligomer species. Note that c-myc and Flag immunoreactivity were not present together in the same higher MW band. B. The staggered expression of μ and δ receptors was performed, in which one receptor was expressed 24 h before the expression of the other. Confocal microscopy showed that μOR (mRFP) and δOR (GFP) were colocalized at the plasma membrane. C. No BRET signal was detected between staggered receptors (μ-Rluc//δ-GFP), in contrast to when they were coexpressed (μ-Rluc + δ-GFP). The agonists, DAMGO and deltorphin II, failed to induce any BRET between staggered μOR and δOR. Results are means ± SEM of three independent experiments each representing at least 5 measurments. (*: $p < 0.05$.)

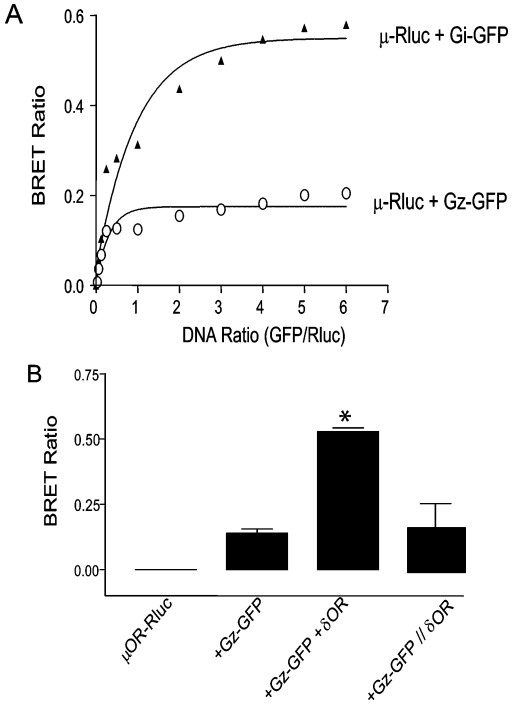

FIGURE 3: Characteristics of the constitutive interaction between μOR and Gi- or Gz-proteins. A. BRET analysis was performed in cells expressing μ-Rluc alone or with either Gz-GFP or Gi-GFP proteins. μ-Rluc DNA amount was maintained constant, whereas the G-protein levels were increased, resulting in saturation-curve BRET ratios. B. BRET analysis of the constitutive interaction between μ-Rluc and Gz-GFP in the presence or in the absence of δ-OR, or when the expression of δ-OR was staggered (//δ-OR). Results are means ± SEM of at least three different experiments each representing 5−10 measurments. (*: $p < 0.05$.)

Figure 2B, left panel, a nonspecific band of ~110 was also seen). In contrast, in the same conditions, the muscarinic M4-GFP receptor was not co-immunoprecipitated with Myc-μOR (Supporting Information Figure 2A, right panel). Furthermore, no co-immunoprecipitation by anti-Myc antibody was observed when δ-GFP was expressed alone. These results suggested that μOR and δOR formed specific heterooligomeric complexes composed of pre-existing homooligomers of each.

To investigate whether the $\mu-\delta$ interaction occurred only in the ER, experiments using staggered expression of receptors were performed, in which one receptor was expressed 24 h before the other receptor was transfected into the same cells. Cells were monitored for receptor coexpression by confocal microscopy and cell surface radioligand binding.

μ-mRFP was expressed in HEK-293 cells 24 h after δ-GFP was expressed, and the live cell images obtained 24 h after the second transfection showed μOR and δOR colocalized at the plasma membrane (Figure 2B). Radioligand binding showed that 74 ± 6% of the steady-state cell surface receptors were produced within 24 h post-transfection (Supporting Information Figure 3). However, no significant BRET signal was detected in cells in which μ-Rluc and δ-GFP expression was staggered, in comparison with cells where μ-Rluc and δ-GFP were cotransfected at the same time (Figure 2C), suggesting that no significant interaction between μ-Rluc and δ-GFP occurred when their expression was staggered, even though both receptors were colocalized.

Table 1: The Interaction of μ-OR with Gz and Gi Proteins in the Presence or the Absence of δ-OR Evaluated by BRET[a]

condition	$BRET_{max}$	$BRET_{50}$
μ-Rluc + Gz-GFP	0.17 ± 0.02	0.94 ± 0.28
μ-Rluc + Gz-GFP + Flag-δ	0.38 ± 0.03	0.91 ± 0.22
μ-Rluc + Gi-GFP	0.50 ± 0.08	<0.01
μ-Rluc + Gi-GFP + Flag-δ	0.14 ± 0.01	nd[b]

[a] BRET analysis was performed in cells expressing μ-Rluc alone (not shown) or with Gz-GFP or Gi-GFP in the presence or the absence of δOR. μ-Rluc and δOR cDNA amounts were maintained constant, whereas the G-protein levels were increased, to obtain saturation-curves of BRET ratios. The analysis showed a switch in μOR coupling to Gz versus Gi, depending on the presence of δOR. Results are means ± SEM of three different experiments, each representing at least 5−10 measurements. [b] Not determined.

Thus, colocalization of the receptors within a specific cellular compartment was not sufficient to predict a physical interaction between them. Further, an interaction could not be induced between the staggered expressed receptors following exposure to either DAMGO or deltorphin II (Figure 2C), showing that the μ-Rluc and δ-GFP interaction was not agonist-triggered and could not be induced at the cell surface, even though both proteins were present there. Thus the formation of $\mu-\delta$ heterooligomers preceded their expression at the plasma membrane; heterooligomerization appeared to be a constitutive process occurring in the ER during receptor biosynthesis and processing, and exposure to agonists at the cell surface was devoid of any significant effect in inducing heterooligomerization.

3. G Protein Preference and Coupling of $\mu-\delta$ Heterooligomers. We previously showed that $\mu-\delta$ heterooligomeric complexes regulated cAMP accumulation through a PTX-insensitive G-protein (*9*) and showed by selective co-immunoprecipitation and GTPγ^{35}S incorporation studies that this PTX-insensitive G-protein was Gz (*18*). In order to investigate this further, BRET experiments were performed on HEK-293 cells transiently expressing μ-Rluc alone or together with Gz-GFP or Gi-GFP, in the presence or in the absence of δOR. Keeping μ-Rluc levels constant, a concentration curve was obtained by varying Gz-GFP or Gi-GFP expression levels (Figure 3A). The $BRET_{max}$ and $BRET_{50}$ values resulting from these curves (Figure 3A and Table 1) suggested a constitutive interaction between μOR and both Gi and Gz, however with a greater preference for Gi over Gz. In contrast, when Gz-GFP was coexpressed both with μ-Rluc and δOR, the $BRET_{max}$ between μOR and Gz was 3-fold greater than in the absence of δOR, and staggering the expression of δOR resulted in the abolition of the increase observed in the presence of δOR (Figure 3B and Table 1). These results suggested that heterooligomerization of $\mu-\delta$ favored robustly the constitutive interaction of the $\mu-\delta$ heteromeric units with Gz-protein. This was also reflected when concentration-dependent effects were tested, where μ-Rluc (and δOR when applicable) DNA concentration was maintained constant while Gi-GFP or Gz-GFP DNA concentrations were increased (Figure 4A and 4B, respectively). The $BRET_{max}$ and $BRET_{50}$ (Table 1) values resulting from these experiments showed that μOR in the absence of δOR interacted preferentially and more robustly with Gi than with Gz, with a relatively higher $BRET_{max}$ of 0.50 versus 0.14 ($p < 0.005$) (Figure 4A and 4B). However, when μOR and δOR were coexpressed, the constitutive interaction with Gz was

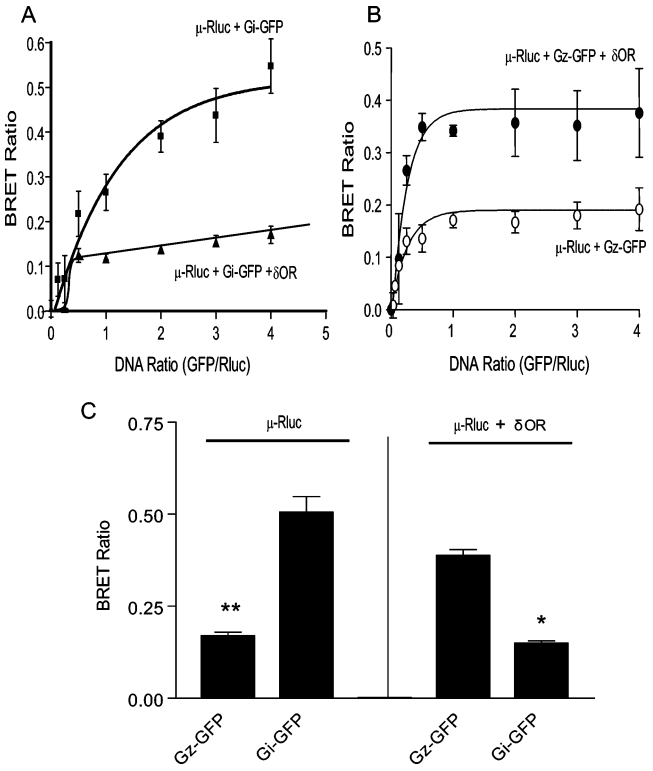

FIGURE 4: Switch in the constitutive coupling between μOR and G-proteins. BRET analysis was performed in cells expressing μ-Rluc alone (not shown) or with Gz-GFP or Gi-GFP in the presence or the absence of δOR. μ-Rluc and δOR cDNA amounts were maintained constant, whereas the G-protein levels were increased, to obtain saturation-curves of BRET ratios. A. Saturation curve of μ-Rluc interaction with Gi-GFP in the presence or the absence of δOR. Note, that the curve is linear in the presence of δOR consistent with a nonspecific interaction. B. Saturation curve of the constitutive interaction between μ-Rluc and Gi-GFP in the presence or the absence of δOR. Values shown are the means and SEM of at least three different experiments each representing 6 measurments. C. Comparison of the maximal BRET between μ-OR and Gi- or Gz-proteins in the presence or the absence of δOR. The results showed a switch in μOR coupling to Gz versus Gi, depending on the presence of δOR. Results are means ± SEM of three different experiments, each representing at least 5−10 measurements. (*: $p < 0.05$. **: $p < 0.01$.)

favored over Gi, with the receptor heteromeric complexes showing higher $BRET_{max}$ and lower $BRET_{50}$ for Gz (Figure 4C and Table 1), reflecting a higher relative affinity for Gz. Although a $BRET_{max}$ of 0.14 (Table 1) between $\mu-\delta$ and Gi was detected, the BRET saturation curve analysis showed a linear, nonsaturable relationship (Figure 4A) and the $BRET_{50}$ was undetermined, indicating that $\mu-\delta$OR heterooligomers had probably no specific interaction with Gi. This suggested a switch in the preference of the constitutive interaction of μOR with these Gα-proteins, likely depending on the intracellular domain configurations within a homooligomeric versus a heterooligomeric complex.

Further, we investigated the subcellular localization of the interactions between the opioid receptors and Gz. HEK 293 cells expressing μ-Rluc, δOR, and Gz-GFP were fractionated on a discontinuous (10−60%) sucrose gradient, and aliquots were subject to BRET analysis (Figure 5A), immunoblotting (Figure 5B), or luminescence and fluorescence measurements (Figure 5C). BRET signals were detected both in the ER (fractions 2−6) and the plasma membrane (fractions 7−13), indicating that μ-Rluc (in the presence of δOR) interacted

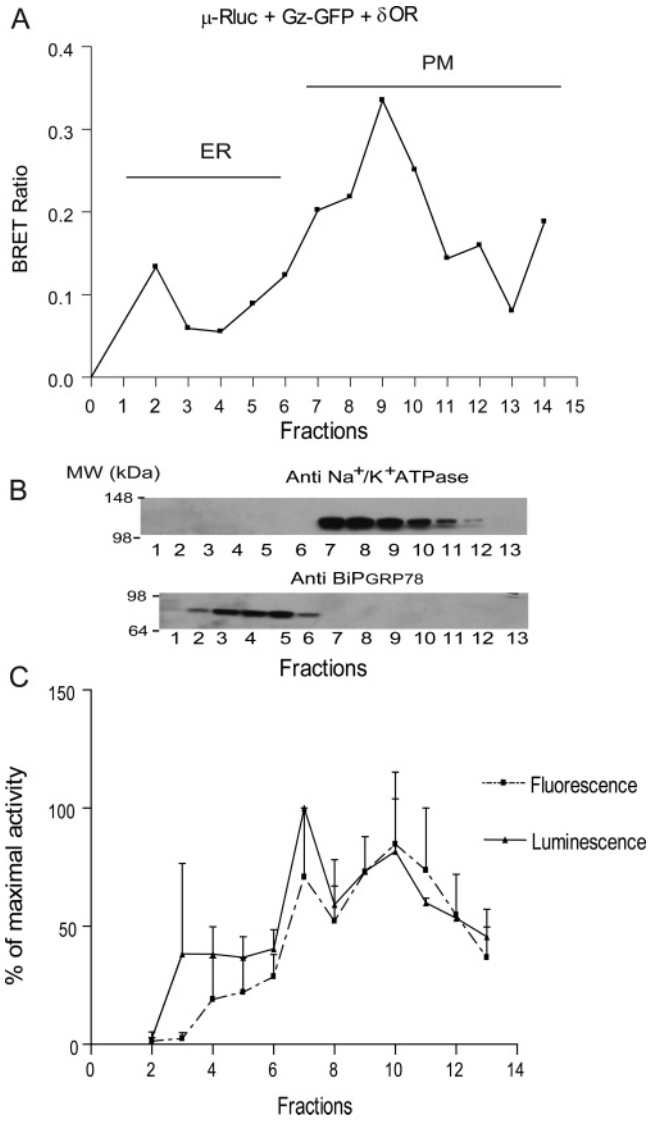

FIGURE 5: Subcellular localization of the interaction between $\mu-\delta$OR and Gz. HEK 293 cells expressing μ-Rluc, Flag-δOR, and Gz-GFP were fractionated on a discontinuous (10−60%) sucrose gradient. A. The fractions were subjected to BRET analysis. BRET signals were detected both in the ER and the plasma membrane (PM) fractions indicating that μ-Rluc (in the presence of δOR) interacted constitutively with Gz-GFP in the ER and this interaction was also present at the plasma membrane. B. The fractions were separated on SDS−PAGE. Immunoblot of fractions using antibodies to Na^+/K^+ ATPase (plasma membrane marker, upper blot) and BiP_{GRP78} (endoplasmic reticulum marker, lower blot). C. Luminescence and fluorescence measurements for each fraction, showing that BRET ratio values in each fraction were in accordance with the expression levels of the receptors (luminescence) and Gz-GFP (fluorescence). Figures are representative of three different experiments.

constitutively with Gz-GFP in the ER and this interaction continued at the plasma membrane. Similarly, BRET results between μ-Rluc and Gi or Gz were obtained whether μ-Rluc was expressed alone or coexpressed with δOR, indicating that opioid receptors interacted with G proteins in the ER/Golgi compartments and continued at the plasma membrane. The BRET ratio values in each fraction were in accordance with the expression levels of the μ-Rluc receptor (luminescence) and Gz-GFP (fluorescence) in each fraction, 48 h post-transfection (Figure 5C).

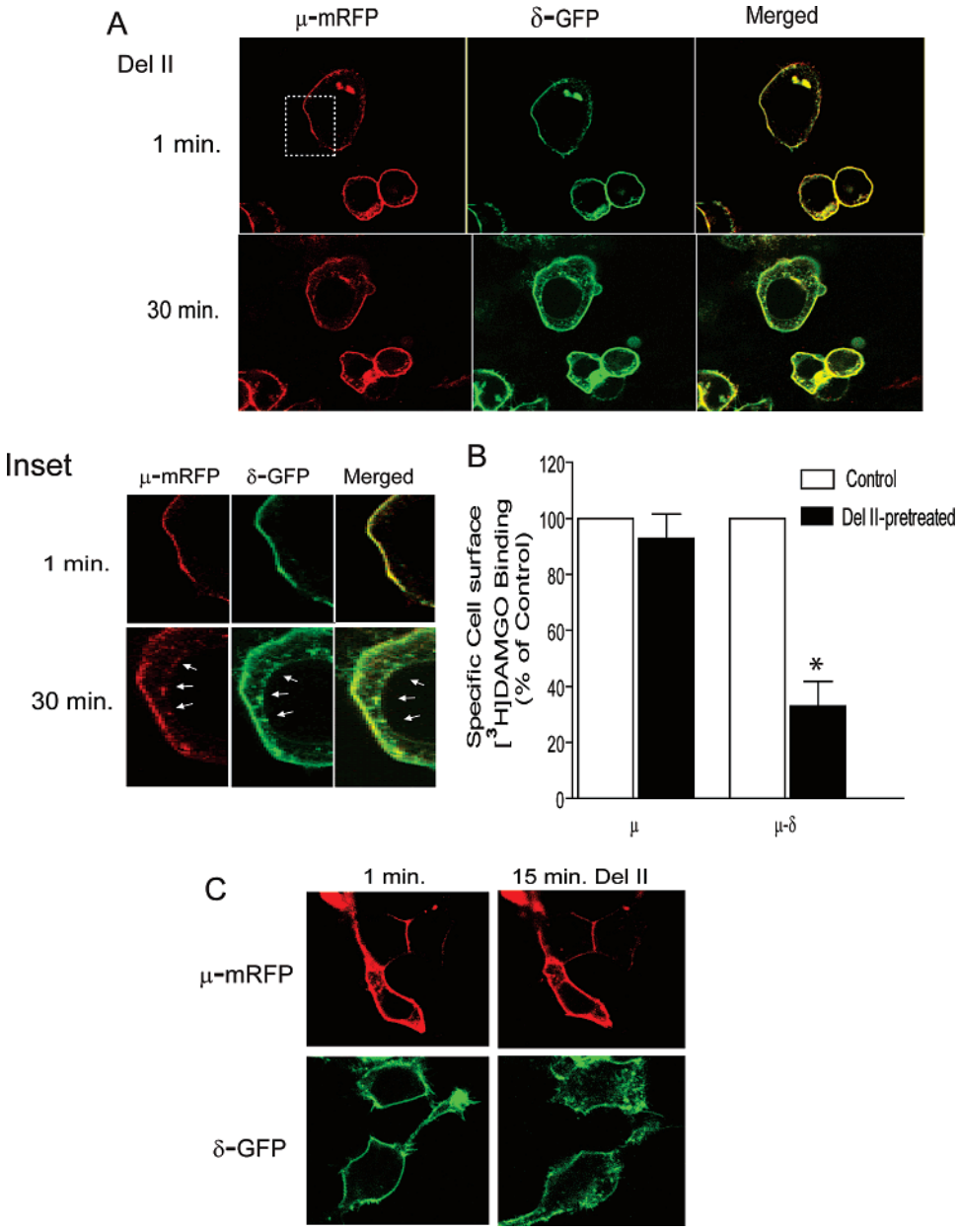

FIGURE 6: Cointernalization of δOR and μOR following deltorphin II treatment. HEK-293 cells expressing μ-mRFP and δ-GFP together or singly were exposed to 100 nM deltorphin II for different times, and monitored by real-time live cell confocal microscopy. A. In cells coexpressing δ-GFP and μ-mRFP, both receptors underwent cointernalization and intracellular colocalization after 30 min exposure to deltorphin II. The inset shows a magnified view of the same cells after Del II treatment. Arrows denote intracellular vesicles containing both receptors. B. Radioligand binding of [^{3}H]DAMGO to whole cells expressing Myc-μOR alone or with Flag-δOR before and after exposure to Del II for 1 h. The density of cell surface μOR was not affected by Del II pretreatment in cells expressing μOR alone, whereas there was a loss of ~70% of cell surface μOR in cells coexpressing μOR and δOR. Results are means ± SEM of three independent experiments, each performed in triplicate. C. Treatment with Del II had no effect on μ-mRFP expressed alone (top panel), whereas it triggered internalization and intracellular localization of δ-GFP expressed alone within 15 min (lower panel). (*: $p < 0.01$.)

4. Agonist-Induced Trafficking of $\mu-\delta$ Heterooligomers Compared to Homooligomers. The effect of $\mu-\delta$ heterooligomerization on the trafficking of each protomer from the cell surface in response to specific agonists was investigated, to determine if selective activation of one receptor had any effect on the other constituent of the heterooligomer, using real time live cell confocal microscopy and whole cell radioligand binding to quantify cell surface receptor densities. When expressed individually, μOR and δOR were shown to undergo agonist-induced internalization in response to their specific agonists, DAMGO and deltorphin II, respectively (*29*−*31*), as well as in response to nonselective agonists such as etorphine (*23*, *32*, *33*).

When μOR and δOR were coexpressed in the same cells, both receptors were colocalized at the cell surface (Figure 6A and inset). Treatment with deltorphin II for 1−15 min had a small effect on both μOR and δOR receptors with a small proportion of the receptors colocalizing intracellularly. However, after 30 min, deltorphin II induced internalization of δ and μOR with intracellular colocalization of both receptors (Figure 6A and inset, 30 min). Both μOR and δOR in the heterooligomeric complexes underwent cointernalization, behaving in response to deltorphin II stimulation as single entities, suggesting a simultaneous coregulation of both oligomeric partners.

Table 2: Agonist-Induced Internalization of μ-OR and δ-OR

receptor	Del II	DAMGO	DPDPE	DSLET
μOR	0	31 ± 3	0	0
δOR	43 ± 5	0	25 ± 4	nd[b]
μOR in $\mu-\delta$ complexes	63 ± 8	45 ± 2	3 ± 2	0
δOR in $\mu-\delta$ complexes	71 ± 9	41 ± 5	6 ± 3	0

[a] Whole cell surface radioligand binding experiments were performed on HEK-293 cells expressing μ-OR, δ-OR, or both μ-OR and δ-OR. Cells were pretreated with 100 nM of the indicated agonist for 1 h prior to the radioligand binding. The amounts of internalized receptors were estimated in comparison to the nontreated cells and expressed as a percentage. Results are means $\pm$ SEM of at least three independent experiments. [b] Not determined.

Estimating cell surface receptor density on whole cells further substantiated these findings. HEK-293 cells transfected with myc-μOR with or without Flag-δOR were treated with 100 nM deltorphin II for 1 h at 37 °C, and radioligand binding was performed on whole cells at 4 °C using cell impermeable [^{3}H]DAMGO 2.5 nM to evaluate μOR density at the cell surface. Exposure to 100 nM deltorphin II had no significant effect on surface density of μOR expressed alone, compared with untreated cells (Figure 6B, open bars). However, in cells coexpresssing μOR and δOR this treatment resulted in the loss of almost 70% ($p < 0.005$) of μOR from the cell surface (Figure 6B, solid bars and Table 2). As controls, in cells expressing μ-mRFP alone, exposure to 100 nM deltorphin II for 1 min or 15 min (Figure 6C, upper panel) showed no difference in μOR localization or appearance of the cells, with receptors remaining at the cell surface, indicating lack of internalization induced by deltorphin II. In cells expressing δ-GFP (Figure 6C, lower panel) following exposure to deltorphin II 100 nM for 1 min, although most of the receptors were at the cell surface, δOR internalization was already initiated, with some receptor movement to the intracellular part of the plasma membrane. After 15 min of deltorphin II exposure, the receptors were significantly localized in the cytoplasm, reflecting robust δ-GFP internalization. Taken together, these results showed that deltorphin II induced not only the internalization of δOR but also triggered the cointernalization of μOR when these receptors were coexpressed, indicating internalization of the $\mu-\delta$ receptor complexes. These results also showed that δOR homooligomers internalized more rapidly than when in a heteromeric complex with μOR.

We also investigated the effects of exposure to 100 nM DAMGO on μOR and δOR when each receptor was expressed alone and when both receptors were coexpressed. The results from radioligand binding studies, aimed to measure cell surface δOR expression before and after DAMGO treatment, are summarized in Table 2. These data showed that while DAMGO had no significant effect on cell surface δOR expressed alone, there was a 55% ($p < 0.005$) reduction of δOR after exposure to DAMGO for 1 h in cells coexpressing μ and δOR. These results were substantiated by confocal microscopy on live cells. μ-mRFP expressed in HEK-293 cells was mainly localized at the cell surface in the absence of any treatment and underwent robust and rapid internalization after treatment with 100 nM DAMGO for 5−10 min, whereas δ-GFP expressed alone was not sensitive to DAMGO, with the receptor mainly localized at the plasma membrane in the absence or the presence of DAMGO (data not shown). However, when μOR and δOR were coexpressed, DAMGO triggered the cointernalization of both receptors (Figure 7). Real-time confocal microscopy analysis showed that after 1 min of exposure to DAMGO a minimal amount of $\mu-\delta$ cointernalization was observed. A robust internalization of both receptors was observed after 45−60 min of exposure as shown in the figure, indicating that this cointernalization was less rapid than that seen for μOR alone (15−20 min), and was accompanied by the colocalization of μ-mRFP and δ-GFP in intracellular compartments. These results, in addition to those obtained with radioligand binding studies (Table 2), confirmed that $\mu-\delta$ heterooligomeric complexes acted as single coregulated entities that could be internalized from the cell surface following the selective activation of either receptor constituent of the heteromeric complex. The differences observed in the time-course of the agonist effects also suggested that the $\mu-\delta$ heterooligomeric complexes were pharmacologically distinct from individual μOR or δOR homooligomers. Other agonists, DPDPE and DSLET, were also tested. Treatment of δOR expressed alone with DPDPE led to robust internalization of this receptor (Figure 8A). However, internalization was abolished in cells coexpressing μ and δORs (Figure 8B), indicating no effect of DPDPE on $\mu-\delta$ heterooligomer internalization, and suggests an agonist-specificity for this process. A similar effect was observed with DSLET, a δ-specific agonist known to induce the internalization of δOR (*29*). Whole cell binding studies showed that pretreatment with 100 nM DSLET had no effect on $\mu-\delta$OR heterooligomers, with both receptors remaining at the cell surface, and with no change in either the Bmax (Table 2) or the affinity (data not shown). These results (with DPDPE and DSLET), indicate that $\mu-\delta$OR cointernalization was an agonist-specific process, and that the heteromeric complexes were co-regulated units, either cointernalizing or remaining at the cell surface in response to different stimuli, indicating a pharmacological profile for agonist-induced internalization of $\mu-\delta$ that was quite distinct from that for μ and δ homooligomeric complexes.

To evaluate the internalization of staggered, colocalized but not interacting, μ and δOR, one receptor was expressed

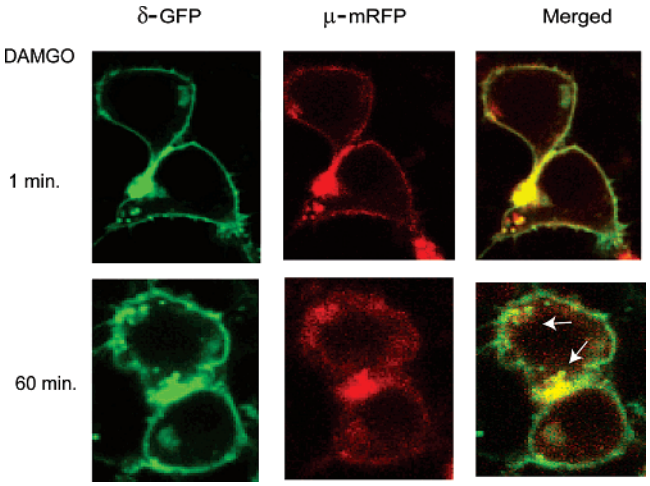

FIGURE 7: Cointernalization of μOR and δOR following DAMGO treatment. HEK-293 cells expressing both μ-mRFP and δ-GFP were exposed to 100 nM DAMGO for different times, and monitored by live cell confocal microscopy in real time. Treatment with DAMGO induced cointernalization and intracellular colocalization of both μ-mRFP and δ-GFP, shown after 60 min of exposure.

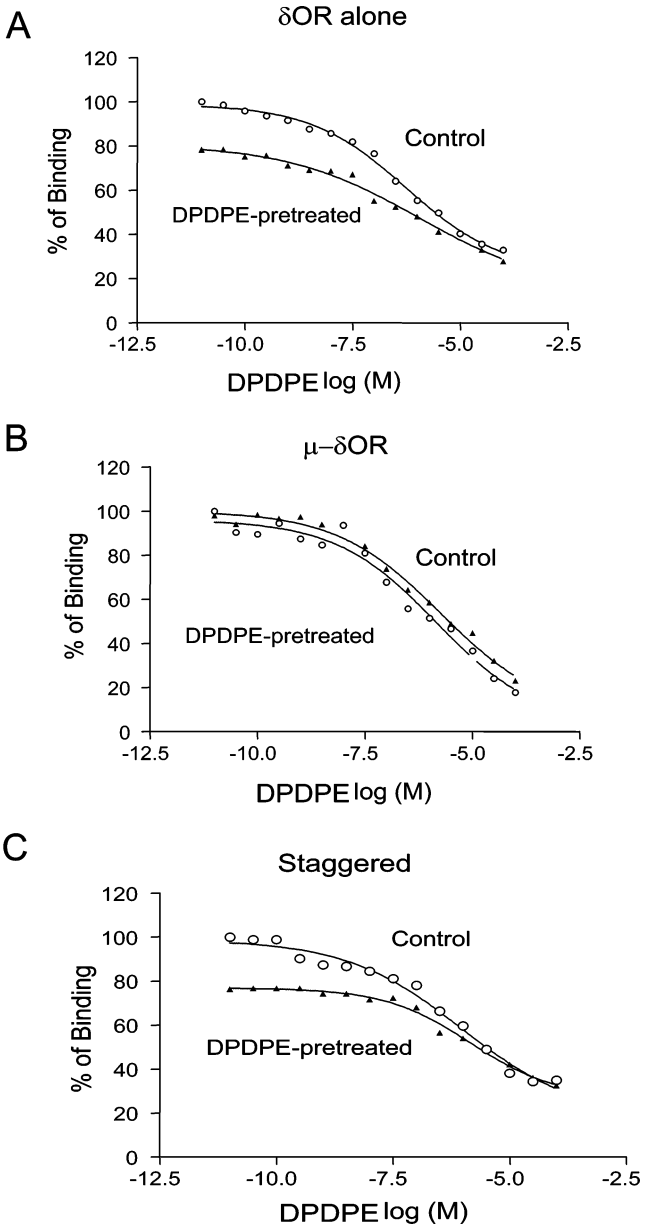

FIGURE 8: Effect of heterooligomerization on DPDPE-mediated internalization of δOR. HEK-293 cells expressing either δOR alone or with μOR were exposed to 100 nM DPDPE for 1 h, and δOR density and affinity were monitored in whole cell displacement binding studies using [^{3}H]naloxone and DPDPE. A. Treatment with DPDPE induced a measurable internalization of δOR expressed alone. B. In cells coexpressing μOR and δOR, DPDPE had no effect on δOR internalization. C. When the expression of μOR and δOR was staggered, DPDPE induced internalization of δOR. Results are representative of three independent experiments, each performed in triplicate.

24 h before the other receptor was transfected. In the absence of any treatment (controls), both receptors expressed thus were colocalized at the cell surface. Exposure to 100 nM deltorphin II for 20 min (not shown) or 60 min (Figure 9A) resulted in a robust internalization of δ-GFP, without affecting μ-mRFP, which was left on the cell surface. Thus, δ-GFP behaved as if it was expressed alone, indicating that μOR and δOR expression, when staggered, did not result in the formation of heterooligomeric complexes and the separate homooligomers remained distinct and did not interact at the cell surface.

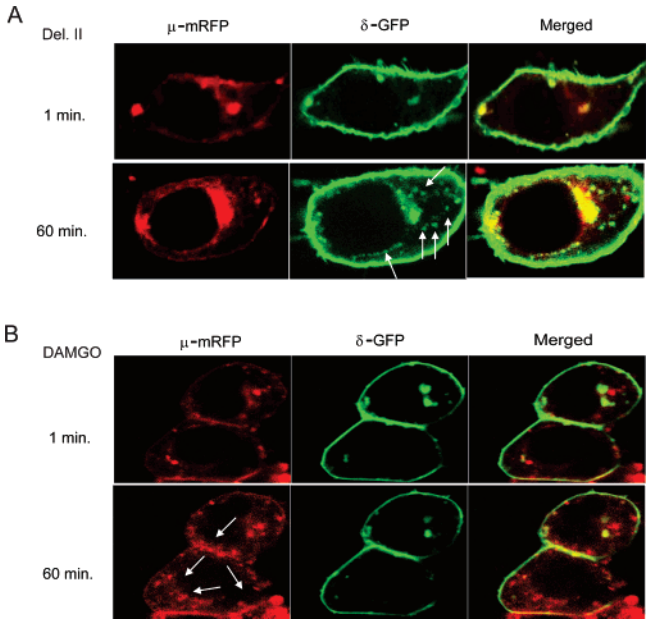

FIGURE 9: Agonist-induced internalization of staggered δOR and μOR indicates non-interacting receptors. μ-mRFP and δ-GFP expression was staggered in HEK-293 cells. Effects of exposure to 100 nM deltorphin II or DAMGO were monitored over time by live cell confocal microscopy. A. Deltorphin II treatment for 60 min resulted in the internalization of δ-GFP but had no effect on μ-mRFP, left on cell surface. B. DAMGO treatment for 60 min resulted in the internalization of μ-mRFP solely, with δ-GFP remaining at the cell surface. Arrows show internalized μ-mRFP or δ-GFP in intracellular vesicles.

Similar observations resulted from DAMGO treatment of μ and δOR expressed in a staggered manner. In this case, only μOR was internalized rapidly in cells expressing staggered μOR and δOR (Figure 9B), whereas δOR remained at the cell surface. Similarly, when μOR and δOR expression was staggered (Figure 8C), δOR underwent internalization with DPDPE, emphasizing the fact that the staggered expression of receptors resulted in the generation of independent homooligomeric receptor complexes.

5. Colocalization of μ and δ Opioid Receptors in Rat Brain. For heterooligomerization to occur in vivo, the anatomic colocalization of both receptor partners is essential. Colocalization of μ- and δ-ORs was investigated in rat brain regions. Immunolabeling of μOR and δOR was observed in pyramidal neurons of the frontal cortex (layers III−V) with many neurons expressing both receptors (Figure 10, upper panel). Within individual neurons, δOR immunolabel was found in the cell soma and throughout the extent of apical dendrites up to at least 300 μm away from the cell body. Immunolabeling for δOR was also found in basilar dendrites. In contrast, μOR labeling was found predominantly within the cell body and only in proximal dendrites. In the striatum (Figure 10, lower panel), μOR was highly concentrated in patches, unlike δOR, which appeared to be expressed in interneurons throughout the striatum. Outside of the striatal patches, μOR labeling was low or barely detectable in neurons expressing δOR. These results showed that the distribution and colocalization of both receptors was brain region specific, with a high degree of colocalization of μOR and δOR within certain neurons, and revealed areas where one receptor was expressed without the other. This suggested

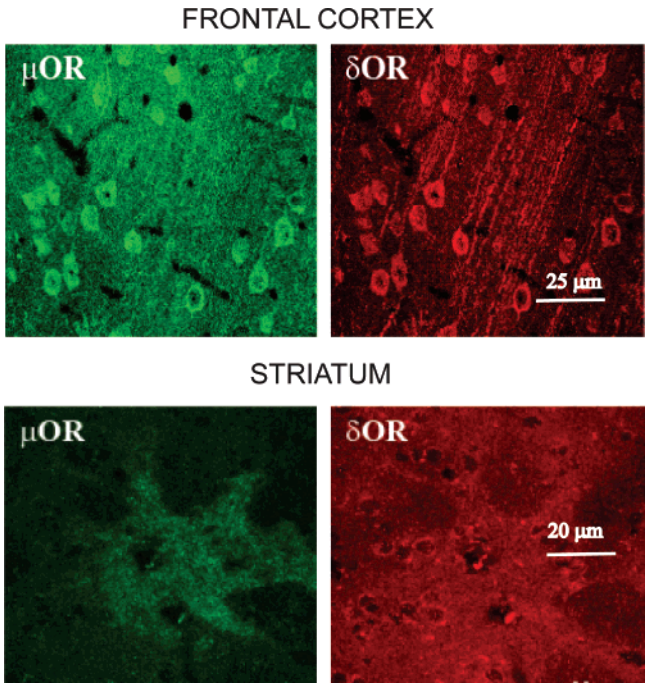

FIGURE 10: Colocalization of μOR and δOR in rat brain. A. Paraformaldehyde-fixed sections (10 μm) from rat brain were incubated with primary antibodies detecting μOR and δOR, then with secondary antibodies linked to FITC or TRITC, and visualized using a Zeiss LSM510 confocal microscope. Immunolabeling was observed in pyramidal neurons of the frontal cortex (layers III−V) with many neurons expressing both μOR and δOR (top panel). In the striatum (lower panel), μOR was highly concentrated in patches, whereas δOR had a more diffuse pattern of expression.

tissue and/or cellular specificity for the physical, and probably functional, interaction.

DISCUSSION

In the present report, we evaluated the subcellular site of μ and δOR heterooligomerization and showed that the receptors interacted constitutively in the ER to form heterooligomeric complexes. Co-immunoprecipitation of δOR by μOR revealed that the heterooligomers were composed of preformed homooligomers, possibly homotetramers. Staggered expression of the receptors resulted in non-interacting populations of μOR and δOR receptors, even though both receptors were colocalized at the cell surface, confirming that $\mu-\delta$OR heterooligomerization had to occur in the ER during receptor biosynthesis or processing, before trafficking to the plasma membrane. We investigated the coupling of the heterooligomers with Gα-proteins, and found that $\mu-\delta$OR heterooligomers showed preference for Gz rather than Gi, with Gz being the major G-protein interacting with the heteromeric complexes. In contrast, μOR homooligomers interacted with much greater affinity and efficiency with Gi. We also showed that the association of the $\mu-\delta$OR heterooligomers with Gα proteins occurred in the ER, before reaching the plasma membrane. These results represent the first indication for the formation in ER-Golgi compartments of heterooligomerized receptors−Gα complexes before their trafficking to the plasma membrane.

We showed that relatively long exposure (35−40 min) to agonists, deltorphin II and DAMGO, led to cointernalization and intracellular colocalization of both receptors, suggesting that the two partners forming the heteromeric complexes internalized as a unit after agonist treatment. However, when receptor expression was staggered, only the receptor targeted by its specific agonist underwent internalization, whereas the other receptor remained at the cell surface, indicating that the receptors were not heterooligomeric and agonist treatment could not induce any *de novo* oligomerization at the cell surface. Time-course experiments showed also that, after agonist activation, μ and δOR homooligomers were internalized faster than the heteromeric complexes. We also showed that deltorphin II was more efficient in triggering the cointernalization of the heterooligomeric complexes than DAMGO.

Only a single δOR band, with a high molecular weight, was co-immunoprecipitated with μOR when both receptors were coexpressed. The antibodies used to reveal μOR did not recognize this band, indicating that this high MW band was exclusively formed of δOR. Further molecular size analysis suggested that the band most probably represents a homotetramer of δOR. The fact that only the homooligomers were detected after co-immunoprecipitation suggested that the heterooligomers are formed of pre-existing homooligomers. This also suggested that the heterooligomers are more sensitive than the homooligomers to SDS and/or β-mercaptoethanol. This could also suggest that the interactions between homooligomers are different and stronger than between the heterooligomers.

In order to avoid any false positive results, which could result from overexpressing the receptors, we performed our experiments at femtomolar receptor densities, not exceeding levels comparable to those observed in native tissues. Under these conditions, BRET saturation studies revealed high $BRET_{max}$ between μ-Rluc and δ-GFP, indicating a robust and specific interaction between μOR and δOR, and low $BRET_{50}$ values, reflecting a high affinity interaction between these receptors. In contrast, under the same conditions μOR did not interact with muscarinic M4 receptor, since no specific BRET was observed between μ-Rluc and M4-GFP, confirming the specificity of μOR−δOR interaction and excluding any experimental artifact effect. Moreover, our findings through BRET analysis of sucrose gradient-fractionated cell lysates showed BRET signals between μOR and δOR not only in plasma membrane-enriched fractions but equally robust in endoplasmic reticulum-enriched fractions, indicating that ER/Golgi is a critical site for the constitutive $\mu-\delta$OR heterooligomerization, before trafficking to the cell surface. Furthermore, the failure of μ and δ receptors expressed in a staggered manner to generate a BRET signal in any subcellular compartment provides compelling evidence for the fact that even though the receptors were coexpressed on the cell surface, they remained discretely separate and could not be induced to oligomerize either by their colocalization on the cell surface or by agonist activation. This provided further conclusive evidence that heterooligomerization only occurred while the receptors were being processed in the ER. It has been shown that some GPCR homooligomers (*10*, *21*, *17*) form in the ER/Golgi, where different trafficking checkpoints of the newly synthesized proteins take place, making the exit from this system an important and crucial step in the regulation of the receptor cell surface expression. The constitutive formation of receptor heterooligomeric complexes in the early stages

of biosynthesis and processing, as we have shown, provides another element contributing to maintaining stringent levels of quality control that scrutinize GPCR oligomer conformation in the ER, before translocation to the plasma membrane. This has been substantiated only for homooligomers as we recently demonstrated that the proper conformational arrangement of D1 dopamine receptor homooligomers was a critical determinant of their ability to traffic to the cell surface (*34*). No evidence for such a regulation for GPCR heterooligomers has been demonstrated, and the only study covering $\mu-\delta$ heterooligomers previously concluded that these complexes were formed only at the cell surface with no alteration in Gi coupling, and each receptor internalized individually in response to agonist stimulation (*35*). We here show that in order for μ and δ heterooligomers to form, the receptors need to be synthesized concurrently, and staggering their expression results in two non-interacting populations of receptors at the cell surface. Our present conclusions differ from those reported by Law et al. (*35*) regarding the site of $\mu-\delta$ receptor oligomerization, G-protein coupling, and agonist-induced trafficking, and suggest that their model generated non-interacting populations of receptors, similar to our results obtained from staggered experiments, which could represent an explanation of the differences between the two studies.

We previously reported that unlike μOR or δOR homooligomers, which couple to Gi, the coupling of $\mu-\delta$OR heteromeric complexes was to G-proteins that were insensitive to pertussis toxin (PTX) (*9*) and subsequently showed that Gz could be activated and co-immunoprecipitated with the heteromeric $\mu-\delta$OR complexes (*18*). Our present BRET studies showed clearly that Gz interacted constitutively with $\mu-\delta$OR complexes with efficiency at least 3-fold greater than with μOR homooligomers. The preference of $\mu-\delta$OR was higher for Gz, whereas μOR homooligomers interacted constitutively with Gi proteins preferentially. This indicates a switch in G-protein coupling based on the configuration of the receptors, i.e., based on their existence within homooligomeric versus heterooligomeric complexes. These data provide novel insight for the first time, into the mechanism of altered affinity of a GPCR for a G-protein based on the identity of the oligomerization partner of the GPCR. Our data also point out that not only are the heterooligomers formed within ER/Golgi but also the interaction of these receptors with Gz and/or Gi occurred at that level, before trafficking as a whole complex to the cell surface. This represents the first data showing total assembly of heterooligomeric receptors with G-proteins in the ER, and is in good agreement with recent data showing that the assembly of β_2-adrenergic receptor homooligomers with $G_{\beta\gamma}$, then G_α, occurred in the ER/Golgi (*36*). An increasing body of data is in favor of the interaction between GPCR-G proteins (*37*), and effectors (*38*) occurring during biosynthesis, and not, as was thought previously, as a result of cell surface random collisions among proteins.

We also investigated the effects of agonist exposure to show cointernalization and intracellular colocalization of both heterooligomeric partners by DAMGO and deltorphin II. These results were substantiated by whole cell radioligand binding experiments, which documented receptor density changes at the cell surface. The intracellular colocalization of $\mu-\delta$OR complexes in our studies suggested that the heterooligomeric complexes were internalized intact in response to agonist. Indeed, the confocal microscopy data using staggered expression of the receptors showed clearly that cointernalization and intracellular colocalization of the receptor partners occurred only when the receptors were coexpressed at the same time. This validates our contention that μOR and δOR interacted to form heterooligomeric complexes constitutively while being processed in the ER. Agonist activation had no role in the oligomerization process per se and did not induce oligomerization de novo at the cell surface, but triggered the cointernalization of the $\mu-\delta$OR complexes. When expressed alone or in staggered experiments, δOR homooligomers underwent faster deltorphin II-induced internalization than when coexpressed with μOR. Similar observations were seen for μOR homooligomers in response to DAMGO treatment. Further, deltorphin II was more efficient than DAMGO in triggering the cointernalization of the heterooligomeric complexes. In contrast, DSLET and DPDPE were unable to induce internalization of δOR coexpressed with μOR, even though δOR homooligomers were robustly internalized, indicating agonist-specific effects on heterooligomer internalization. Taken together, these results suggest that the $\mu-\delta$ heterooligomeric complexes functioned as coregulated units, which were pharmacologically distinct from either μOR or δOR homooligomers. These findings demonstrated a novel regulatory mechanism that could modulate internalization of a given receptor by activating its heteromeric partner.

Both μ- and δOR are important in opioid addiction and analgesia and show functional synergism and pharmacological cooperativity. With the evidence from various cell-based methodologies that these receptors form heterooligomers, it is highly likely that an interaction between these receptors might occur in brain. The occurrence and functional consequences of $\mu-\delta$ heterooligomerization in native tissues will be important to establish. The anatomic colocalization of both receptors in individual neurons as we have shown here represents an essential prerequisite. Extensive $\mu-\delta$OR colocalization was also observed in several regions of rat brain (*39*), as well as in neuroblastoma cell lines (*40*) and in rat and guinea pig enteric neurons (*41*, *42*). Although a broad colocalization has been described, further studies will be necessary to identify the density and distribution of $\mu-\delta$ heterooligomers in brain and elucidate their functional significance.

In summary, the specific and constitutive interaction among μOR, δOR, and Gα-proteins occurred in the ER and resulted in the formation of novel heteromeric signaling units, pharmacologically distinct from the individual homooligomers, with a switch in the Gα-protein coupling preference and altered rank order of agonist-induced internalization. These opioid receptor−G-protein heteromeric signaling complexes have novel implications for GPCR biology with potential to identify new drug targets other than those based on the individual properties of the constituent receptors. It would be also of high interest to determine the specific physiological functions mediated by opioid receptor heterooligomers compared to homooligomers, including agonist-induced activation, regulation, and intracellular signaling events occurring in native tissues in relation to opioid action as well as in tolerance and dependence.

SUPPORTING INFORMATION AVAILABLE

Three additional figures as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Kieffer, B. L. (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides, *Cell. Mol. Neurobiol. 15*, 615−635.
2. Kieffer, B. L., and Evans, C. J. (2002) Opioid tolerance-in search of the holy grail, *Cell 108*, 587−590.
3. Levac, B. A., O'Dowd, B. F., and George, S. R. (2002) Oligomerization of opioid receptors: generation of novel signaling units, *Curr. Opin. Pharmacol. 2*, 76−81.
4. Fowler, C. J., and Fraser, G. L. (1994) Mu-, delta-, kappa-opioid receptors and their subtypes. A critical review with emphasis on radioligand binding experiments, *Neurochem. Int. 24*, 401−426.
5. George, S. R., O'Dowd, B. F., and Lee, S. P. (2002) G-protein-coupled receptor oligomerization and its potential for drug discovery, *Nat. Rev. Drug Discovery 1*, 808−820.
6. Bouvier, M. (2001) Oligomerization of G-protein-coupled transmitter receptors, *Nat. Rev. Neurosci. 2*, 274−286.
7. Milligan, G. (2004) G protein-coupled receptor dimerization: function and ligand pharmacology, *Mol. Pharmacol. 66*, 1−7.
8. Jordan, B. A., and Devi, L. (1999) G-protein-coupled receptor heterodimerization modulates receptor function, *Nature 399*, 697−700.
9. George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., and O'Dowd, B. F. (2000) Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties, *J. Biol. Chem. 275*, 26128−26135.
10. Terrillon, S., Durroux, T., Mouillac, B., Breit, A., Ayoub, M. A., Taulan, M., Jockers, R., Barberis, C., and Bouvier, M. (2003) Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis, *Mol. Endocrinol. 17*, 677−691.
11. Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca, A. J., O'Dowd, B. F., and George, S. R. (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal, *J. Biol. Chem. 279*, 35671−35678.
12. Pascal, G., and Milligan, G. (2005) Functional complementation and the analysis of opioid receptor homodimerization, *Mol. Pharmacol. 68*, 905−915.
13. O'Dowd, B. F., Ji, X., Alijaniaram, M., Rajaram, R. D., Kong, M. M., Rashid, A., Nguyen, T., and George, S. R. (2005) Dopamine receptor oligomerization visualized in living cells, *J. Biol. Chem. 280*, 37225−37235.
14. Gomes, I., Filipovska, J., Jordan, B. A., and Devi, L. A. (2002) Oligomerization of opioid receptors, *Methods 27*, 358−365.
15. Charles, A. C., Mostovskaya, N., Asas, K., Evans, C. J., Dankovich, M. L., and Hales, T. G. (2003) Coexpression of delta-opioid receptors with mu receptors in GH3 cells changes the functional response to mu agonists from inhibitory to excitatory, *Mol. Pharmacol. 63*, 89−95.
16. Ramsay, D., Kellett, E., McVey, M., Rees, S., and Milligan, G. (2002) Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences, *Biochem. J. 365*, 429−440.
17. Wang, D., Sun, X., Bohn, L. M., and Sadee, W. (2005) Opioid receptor homo- and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer, *Mol. Pharmacol. 67*, 2173−2184.
18. Fan, T., Varghese, G., Nguyen, T., Tse, R., O'Dowd, B. F., and George, S. R. (2005) A role for the distal carboxyl tails in generating the novel pharmacology and G protein activation profile of mu and delta opioid receptor hetero-oligomers, *J. Biol. Chem. 280*, 38478−38488.
19. Matthes, H. W., Smadja, C., Valverde, O., Vonesch, J. L., Foutz, A. S., Boudinot, E., Denavit-Saubie, M., Severini, C., Negri, L., Roques, B. P., Maldonado, R., and Kieffer, B. L. (1998) Activity of the delta-opioid receptor is partially reduced, whereas activity of the kappa-receptor is maintained in mice lacking the mu-receptor, *J. Neurosci. 18*, 7285−7295.
20. Kieffer, B. L., and Gaveriaux-Ruff, C. (2002) Exploring the opioid system by gene knockout, *Prog. Neurobiol. 66*, 285−306.
21. Salahpour, A., Angers, S., Mercier, J. F., Lagace, M., Marullo, S., Bouvier, M. (2004) Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting, *J. Biol. Chem. 279*, 33390−33397.
22. Herrick-Davis, K., Weaver, B. A., Grinde, E., and Mazurkiewicz, J. E. (2006) Serotonin 5-HT2C receptor homodimer biogenesis in the endoplasmic reticulum: real-time visualization with confocal fluorescence resonance energy transfer, *J. Biol. Chem. 281*, 27109−27116.
23. Hasbi, A., Allouche, S., Sichel, F., Stanasila, L., Massotte, D., Landemore, G., Polastron, J., and Jauzac, P. (2000) Internalization and recycling of delta-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism, *J. Pharmacol. Exp. Ther. 293*, 237−247.
24. Marie, N., Lecoq, I., Jauzac, P., and Allouche, S. (2003) Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists, *J. Biol. Chem.* ***278***, 22795−22804.
25. Hasbi, A., Devost, D., Laporte, S. A., and Zingg, H. H. (2004) Real-time detection of interactions between the human oxytocin receptor and G protein-coupled receptor kinase-2, *Mol. Endocrinol. 18*, 1277−1286.
26. Yu, J. Z., and Rasenick, M. M. (2002) Real-time visualization of a fluorescent G(alpha)(s): dissociation of the activated G protein from plasma membrane, *Mol. Pharmacol. 61*, 352−359.
27. Bunemann, M., Frank, M., and Lohse, M. J. (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation, *Proc. Natl. Acad. Sci. U.S.A. 100*, 16077−16082.
28. Angers, S., Salahpour, A., and Bouvier, M. (2001) Biochemical and biophysical demonstration of GPCR oligomerization in mammalian cells, *Life Sci. 68*, 2243−2250.
29. Arden, J. R., Segredo, V., Wang, Z., Lameh, J., and Sadee, W. (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells, *J. Neurochem. 65*, 1636−1645.
30. Law, P. Y., Kouhen, O. M., Solberg, J., Wang, W., Erickson, L. J., and Loh, H. H. (2000) Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor, *J. Biol. Chem. 275*, 32057−32065.
31. He, L., Fong, J., Von, Zastrow, M., and Whistler, J. L. (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization, *Cell 108*, 271−282.
32. Chu, P., Murray, S., Lissin, D., and von Zastrow, M. (1997) Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist, *J. Biol. Chem. 272*, 27124−27130.
33. Bot, G., Blake, A. D., Li, S., and Reisine, T. (1997) Opioid regulation of the mouse delta-opioid receptor expressed in human embryonic kidney 293 cells, *Mol. Pharmacol. 52*, 272−281.
34. Kong, M. M., Fan, T., Varghese, G., O'Dowd, B. F., and George, S. R. (2006) Agonist-induced cell surface trafficking of an intracellularly sequestered D1 dopamine receptor homo-oligomer, *Mol. Pharmacol. 70*, 78−89.
35. Law, P. Y., Erickson-Herbrandson, L. J., Zha, Q. Q., Solberg, J., Chu, J., Sarre, A., and Loh, H. H. (2005) Heterodimerization of mu- and delta-opioid receptors occurs at the cell surface only and requires receptor-G protein interactions, *J. Biol. Chem. 280*, 11152−11164.
36. Dupré, D. J., Robitaille, M., Ethier, N., Villeneuve, L. R., Mamarbachi, A. M., and Hébert, T. E. (2006) Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking, *J. Biol. Chem. 281*, 34561−34573.
37. Galés, C., Rebois, R. V., Hogue, M., Trieu, Ph., Breit, A., Hébert, T.E., and Bouvier, M. (2005) Real-time monitoring of receptor and G-protein interactions in living cells, *Nat. Methods 2*, 177−184.
38. Lavine, N., Ethier, N., Oak, J. N., Pei, L., Liu, F., Trieu, Ph., Rebois, R. V., Bouvier, M., Hébert, T. E., and Van To, H. H. M (2002) G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase, *J. Biol. Chem. 277*, 46010−46019.

39. Gray, A. C., Coupar, I. M., and White, P. J. (2006) Comparison of opioid receptor distributions in the rat central nervous system, *Life Sci. 79*, 674−685.
40. Baumhaker, Y., Wollman, Y., Goldstein, M. N., and Sarne, Y. (1993) Evidence for mu-, delta-, and kappa-opioid receptors in a human neuroblastoma cell line, *Life Sci.* 52, PL205−PL210.
41. Egan, T. M., and North, R. A. (1981) Both mu and delta opiate receptors exist on the same neuron, *Science 214,* 923−924.
42. Gray, A. C., Coupar, I. M., and White, P. J. (2005) Comparison of opioid receptor distributions in the rat ileum, *Life Sci. 78*, 1610−1616.

BI701436W