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Differential Response to Morphine of the Oligomeric State of μ-Opioid in the Presence of δ-Opioid Receptors

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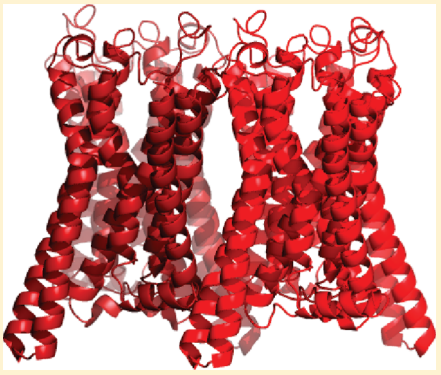
Supporting Information

ABSTRACT: Prolonged morphine treatment induces extensive desensitization of the μ-opioid receptor (μOR) which is the G-protein-coupled receptor that primarily mediates the cellular response to morphine. To date, the molecular mechanism underlying this process is unknown. Here, we have used live cell fluorescence imaging to investigate whether prolonged morphine treatment affects the physical environment of μOR, or its coupling with G-proteins, in two neuronal cell lines. We find that chronic morphine treatment does not change the amount of enhanced yellow fluorescence protein (eYFP)-tagged μOR on the plasma membrane, and only slightly decreases its association with G-protein subunits. Additionally, morphine treatment does not have a detectable effect on the diffusion coefficient of eYFP-μOR. However, in the presence of another family member, the δ-opioid receptor (δOR), prolonged morphine exposure results in a significant increase in the diffusion rate of μOR. Number and brightness measurements suggest that μOR exists primarily as a dimer that will oligomerize with δOR into tetramers, and morphine promotes the dissociation of these tetramers. To provide a plausible structural context to these data, we used homology modeling techniques to generate putative configurations of μOR-δOR tetramers. Overall, our studies provide a possible rationale for morphine sensitivity.



Opioid receptors are seven-transmembrane (TM) G-protein-coupled receptors (GPCRs). They can be classified into three major subfamilies, μOR, δOR, and κOR, which mediate different physiological functions and which vary in their tissue distribution. With the exception of a few selective compounds, all opioid receptors are capable of binding to the same endogenous ligands, such as dynorphins, enkephalins, endorphins, as well as the same exogenous ligands, such as morphine and other analgesics.[1] Opioid receptors bind to these different ligands with different affinities, thus generating varying cellular responses.

Opioid receptors are seven-transmembrane (TM) G-protein-coupled receptors (GPCRs) and are coupled to the $G\alpha_i$ family of heterotrimeric G-proteins. The main cellular effect of $G\alpha_i$ activation is inhibition of adenylyl cyclase resulting in reduced levels of intracellular cAMP (for reviews, see refs 2−4). In general, after a ligand binds to its specific GPCR, the receptor is phosphorylated by a receptor kinase, where it then clusters on the membrane surface and internalizes into endosomes to quench the signal. However, this is not the case for morphine stimulation of μOR. Prolonged treatment of cells with morphine diminishes its elicited response without significant internalization (i.e., no significant decrease in the amount of receptor on the plasma membrane). The mechanism that underlies this unique desensitization behavior is not well understood but appears to be a combination of many cellular events. Desensitization may involve changes in proteins that regulate receptor phosphorylation and internalization[1] and may also involve decoupling between receptors and G-proteins.[5]

Opioid receptors, like other GPCRs, appear to associate into dimers and higher order oligomers which may alter their ligand binding and G-protein activation.[6,7] Coimmunoprecipitation and bioluminescence resonance energy transfer studies suggest that μOR physically associates with δOR when the two receptors are coexpressed,[8,9] although recent studies in mice suggest the two receptors may have distinct localization and activators.[10] It has been found that chronic morphine upregulates μOR-δOR dimers[11] and that activation of the δOR subunit of δOR-μOR dimers leads to increased μOR degradation and a reduced cellular response.[12] The mechanism that underlies this enhanced degradation is unclear.

Here, we report on the changes in the interaction between μOR and its attached G-proteins following prolonged morphine

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treatment, and the influence of δOR on these interactions. We used Förster resonance energy transfer (FRET) to measure changes in association between receptors and G-proteins, fluorescence correlation spectroscopy (FCS) to measure mobility of the receptors and G-protein subunits, and number and brightness (N&B) analysis to monitor the degree of receptor oligomerization. Additionally, we used computational modeling to offer a structural interpretation of the experimental data.

We find that morphine has little effect on the diffusion properties of μOR alone, or its interaction with G-proteins. However, in the presence of δOR, morphine treatment affects the extent of oligomerization of μOR, as well as the association of μOR with G-proteins. Taken together, these findings show how the functional properties of μOR are correlated with its oligomerization with δOR.

MATERIALS AND METHODS

Materials. Fluorescent-labeled opioid receptors have been previously described.[13] Fluorescent-tagged G-proteins were a gift from Catherine Berlot (Gesinger Institute, Lewisburg, PA) and have been well characterized,[14−17] and the double eGFP construct was a gift from Dr. Enrico Gratton (Laboratory of Fluorescence Dynamics, University of California, Irvine).

Cell Culture and Transfection. HEK293 and SK-N-SH cells were grown in modified Eagle's medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 50 units/mL of penicillin and 50 μg/mL of streptomycin sulfate. Neuro2a cells were grown in DMEM and F12 media (50:50) supplemented with 10% FBS and 50 units/mL of penicillin and 50 μg/mL of streptomycin sulfate. Cells were maintained at 37 °C in a 5% CO_2 incubator. Neuro-2a and SK-N-SH cells were transfected using Lipofectamine per the manufacturer's protocol (Invitrogen). HEK-293 cells were transfected using the calcium phosphate coprecipitation method. For FRET measurements, cells were transfected with 5 μg of enhanced yellow fluorescent protein (eYFP)-μOR, 5 μg of eCFP-Gβ1 and 10 μg of HAGγ7 or 5 μg of enhanced cyan fluorescent protein (eCFP)-μOR, and 5 of μg eYFP-Gαi. We have previously estimated that this amount of DNA results in a 3-fold amount of overexpressed overendogenous G-protein subunits in HEK293 cells.[18] Cells expressing low amounts of proteins were selected for viewing, and we note that FCS and N&B measurements can only be done for ≥20 fluorescent molecules. For FCS and N&B measurements, cells were transfected with 2 μg of enhanced green fluorescent protein (eGFP)-μOR and, when noted, with 5 μg of myc tagged δOR. Prior to fluorescence measurements, transfected cells were washed and imaged in phenol free Libovitz 15 media.

In Vivo Single Cell FRET Measurements. FRET experiments were performed on Zeiss LSM 510 Meta/confocor2 apparatus (Jena, Germany) using the synthesized emission method and have been previously described.[19] We used a 40× NA 1.2 C-Apochromat water immersion objective and the following filter settings: eCFP excitation, 458 nm line of argon ion laser; and emission, 475−525 nm band-pass filter; eYFP excitation, 514 nm line of argon ion laser; and emission, 560−615 nm band-pass filter; and FRET excitation, 458 nm; and emission, 560−615 nm band-pass filter. Bleed-through from eCFP fluorescence into the FRET channel and direct excitation of eYFP by the 458 nm laser line values were estimated from cells transfected with eYFP-μOR and eCFP-Gβ1/HAGγ7 separately and imaged under the appropriate filter sets. The maximum FRET value was determined from control cells transfected with the construct composed of eCFP and eYFP sandwiched between a 12 amino acid peptide.

After background subtraction, NFRET values were calculated for every pixel in the image according to the following formula:

$$NFRET = \frac{I_{FRET} - a \times I_{YFP} - b \times I_{CFP}}{\sqrt{I_{YFP} \times I_{CFP}}}$$

where a is the percentage of bleed-through of eCFP through the FRET filter set, and b is the percentage of direct excitation of eYFP by 458 nm light.[20]

Fluorescence Correlation Spectroscopy Measurements. FCS measurements were performed on a Zeiss LSM 510 Meta/Confocor 2 apparatus (Jena, Germany) using standard configurations and minimal laser powers to avoid photobleaching of the fluorescent probes. All measurements were performed at room temperature. We used a 40× NA 1.2 C-Apochromat water immersion objective and adjusted the pinholes at least daily. We excited eGFP with the 488 nm line of argon ion laser and collected emission spectra through a 505 LP filter. We calibrated the detection volume by measuring the diffusion of rhodamine (Rh6G, $D = 4.2 \times 10^{-6}$ cm^2/s) in water (Rutinger 2008, Petrasek 2008,). The radius of the detection volume for the 488 nm line was $r = 0.17 \pm 0.01$ μm. We rejected measurements that showed abrupt and significant changes in the count rate to avoid artifacts due to bleaching and/or cell movement. We used Sigma Plot and a least-squares algorithm to fit the autocorrelation curves to the model equation for free Brownian diffusion in two dimensions commonly used in FCS:[21]

$$G(\tau) = \frac{1}{N} \cdot \sum_i \frac{Y_i}{1 + \frac{\tau}{\tau_{d,i}}}$$

where N is number of molecules in the detection volume, Y_i is a fraction of molecules diffusing with diffusion coefficient D_i producing residence times $\tau_{d,i} = r^2/4D_i$. We calculated the diffusion coefficient, D, from the Einstein relationship as follows:

$$D = \frac{r^2}{4\tau_d}$$

Number and Brightness Measurements. Confocal images were collected on an Olympus Fluoview 1000 LSCM fitted with a 60× PlanApo (1.40 NA) oil immersion objective. The analog/digital hybrid detector (PMT) was used in photon counting mode with 1× gain and 0% offset. Images of cells were collected with resolutions of 46 nm/pixel. A region of interest (256 × 256 box) was analyzed from an image of 512 × 512 pixels. The pixel dwell time was 12.5 μs/pixel, and the pinhole diameter was 200 μm. eGFP was excited with the 0.1% of 488-nm line of a 40 mW argon ion laser. In general, 100 images of one cell were collected. Data analysis was done using the N&B analysis screen of the SimFCS program (www.lfd.uci.edu).[22] The offset and the readout noise were determined from the histograms of dark count performed after every measurement (100 images collected with the laser turned off).

Statistical Analysis. We used software provided by Zeiss for FRET and FCS analysis. For number and brightness analysis, we used the SimFCS program described above. For statistical analysis we used SigmaStat (SPSS, INC., Chicago, IL). We compared the values of diffusion coefficient of fluorescent lipids using Kruskal−Wallis One Way Analysis of Variance on Ranks,

Dunn's Method, One Way Analysis of Variance, Tukey's Method, and paired t test. We concluded that the values are significantly different when $P < 0.05$.

Computational Modeling of Heteromeric Complexes of Opioid Receptors. There are no crystal structures of the opioid receptors in either active or inactive states available to date. Thus, assuming that morphine preferentially induces a conformational active state of μOR, we were prompted to use computational modeling techniques to generate all-atom models of the *Mus musculus* μOR in an active conformation and the *Mus musculus* δOR in an inactive conformation. Specifically, an inactive conformation of δOR was obtained using a combination of homology and ab initio modeling for the TM (based on the β2 adrenergic receptor crystal structure corresponding to PDB 2RH1[23]) and loop regions, respectively. Complete details of the modeling strategy and software used to produce these model structures have been reported elsewhere.[24] For the active μOR conformation, a similar combined homology and ab initio modeling strategy was employed, differing in the choice of the template structure for homology modeling of the TM region. On the basis of the proposed similarity between the recent crystal structures of ligand-free opsin and active GPCR conformations, we used the low-pH opsin crystal structure (PDB: 3CAP[25,26]) as the basis for homology modeling of the TM region of active μOR. Because of the absence of a conserved proline residue in TM1 of μOR, we used the TM1 of the β2 adrenergic receptor crystal structure (PDB: 2RH1[23]) as a structural template for this specific helix. We constructed putative three-dimensional (3D) models of μOR-δOR heteromeric tetramers based on inferences from published experimental data[27] and the experimental results reported here. Specifically, we assumed that μOR-δOR associations preferentially involve helices TM1, TM4, and/or TM5 to generate 3D models of μOR-δOR tetramers that formed symmetric interfaces and complied with the fluorescence data. These models were subjected to energy minimization using the steepest descent algorithm as implemented in GROMACS version 4.0.5,[28] with the Optimized Potentials for Liquid Simulations-All Atom (OPLS-AA) force-field.[29]

RESULTS

Changes in the Association between μOR and G Protein Subunits with Morphine Treatment. To test the current theory that morphine treatment changes the degree of association between μOR and G-protein subunits, we monitored the change in FRET between μOR and G-proteins. It has been shown that eCFP-Gβ1/HA-Gγ7 functions identically to wild type,[14] and we found that morphine treatment of cells expressing eYFP-μOR generates a 10 reduction in ^{3}H-cAMP levels (Calizo et al., unpublished work) showing that the transfected constructs we are using are functional.

We coexpressed eYFP-μOR and eCFP-Gβ1/HA-Gγ7 in two neuronal cell lines, Neuro-2a and SK-N-SH at low levels. As expected, in both cell lines the proteins were primarily localized on the plasma membrane with a small amount in intracellular vesicles (e.g., Figure 1A). The values of FRET were the same in both cell types (0.38 ± 0.04, $n = 23$) and were constant 24 to 72 h after transfection. These FRET values were unchanged when the proteins were coexpressed with unlabeled Gα_i. Even though the value of FRET is independent of protein concentration, we choose cells expressing a low and similar amount of the eCFP and eYFP constructs, although the values were found to within error

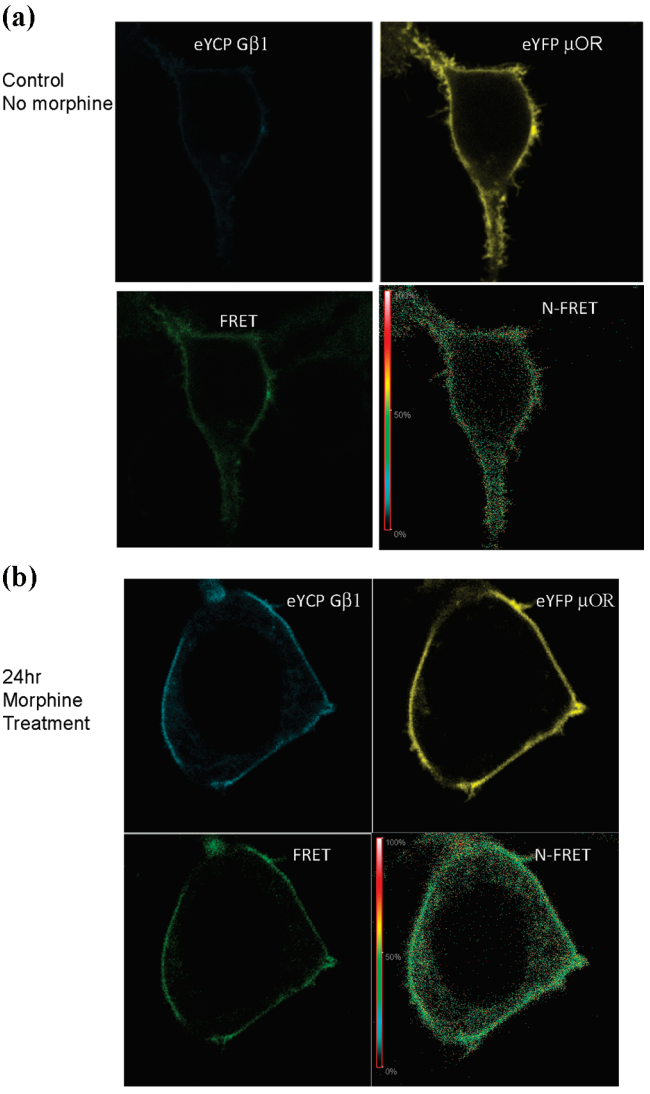

Figure 1. Example of images of eCFP-Gβ1 (top left) and eYFP-μOR (top right), their raw FRET (bottom left) and their normalized FRET (bottom right) in Neuro-2a cells. Images are artificially colorized by Zeiss software.

of each other over an approximate 4 fold range of expression level.

To better understand the values of FRET described above, we measured FRET for a positive control of eCFY and eYFP attached at opposite ends of a dodecameric peptide (0.80 ± 0.03), and a negative control of free CFP and free YFP molecules (0.10 ± 0.02) expressed in cells.[30] Identical low FRET values were obtained for two noninteracting membrane bound proteins (i.e., eCFP-Gαq and PLCδ1,[30] and eCFP-PI3K and eYFP-PLCβ1[19]). Thus, the FRET values seen for μOR and Gβ are significant. When we consider that the labeled proteins must compete with endogenous proteins, then our FRET measurements suggest a high level of association. We note that since we are viewing many cells over a period of time, we express FRET as a value that considers variations in the eCFP and eYFP expression (i.e., NFRET, see Materials and Methods). Note that this value only refers to FRET that is normalized to the intensity levels of the donor and acceptor rather than normalizing the raw FRET to high and low values.

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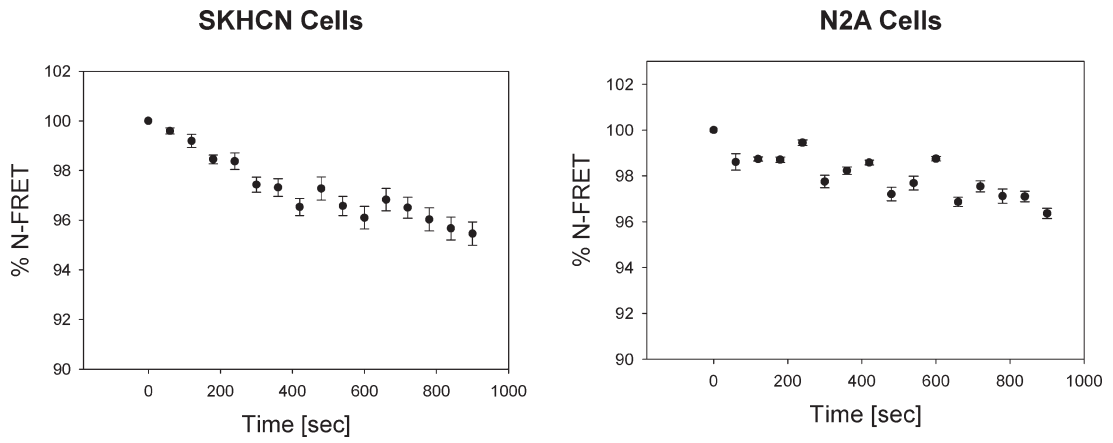

Figure 2. Percent decrease in eCFP-Gβ1 and eYFP-μOR FRET with continuous 1 μM morphine treatment where the values were normalized to 0.038 for SKNSN and 0.036 for Neuro-2a cells.

We determined the response of FRET between eYFP-μOR and eCFP-Gβ1/HA-Gγ7 in Neuro-2a and SK-N-SH with morphine treatment. Continuous treatment with 1 μM morphine over 24 to 72 h did not significantly change in the localization of the proteins or their amount on the plasma membrane as assessed by confocal imaging, supporting the notion that morphine desensitization does not involve a decrease in the amount of receptor on the plasma membrane (Figure1B). To determine whether morphine affects the degree of association between the receptor and G-protein, we measured the change in FRET between eYFP-μOR and eCFP-Gβ1/HA-Gγ7 with morphine treatment. We found a small, but significant, 10% decrease in FRET in both cell lines (Figure 2). While these data show that morphine treatment does not result in large changes in receptor−G-protein interactions, they do imply a relatively small change in receptor G-protein coupling or the local conformation and/or arrangement of the receptor−G-protein complex. These results contradict the idea that the desensitization to morphine tolerance is due to physical uncoupling between μOR and G-protein subunits.

Changes in μOR Oligomerization with Morphine Treatment. Since morphine does not appear to affect the association between μOR and G-proteins, we determined whether it might cause other changes in the physical environment of the receptor. We first assessed changes in the oligomeric state of μOR with morphine treatment using FRET. These studies were carried out by cotransfecting Neuro-2a cells with eCFP-μOR and eYFP-μOR and monitoring changes in FRET with continuous morphine treatment. We found that the receptors displayed a high degree of FRET (0.47 ± 0.04, $n = 12$) that remained constant over 24−72 h of morphine treatment. This value of FRET is in line with what would be expected from a mixed population of homo- and hetero-oligomers of the eCFP and eYFP-tagged μOR. The high value of FRET indicates that a very large population of μOR receptors exists as oligomers.

Many GPCRs appear to reside in higher order lipid and/or protein domains in the plasma membrane which impact their propensity to oligomerize.[18] To determine whether μOR is contained in higher order complexes, we measured its diffusion in Neuro-2a cells using fluorescence correlation spectroscopy (FCS). Free GPCRs are reported to diffuse at a rate of 1×10^{-9} cm^2/s.[31−33] We find that the diffusion coefficient of eYFP-μOR is comparable to these values ($D = 7.3 \pm 0.2 \times 10^{-9}$ cm^2/s), suggesting that the receptor does not localize in large complexes that limit its mobility. Rather, the diffusion data suggest that the receptor is in small oligomers, such as dimers or tetramers. Treatment of the cells with 1 μM morphine for 24 h did not affect the diffusion coefficient of the protein.

Coexpression of δOR Induces Changes in the μOR Environment with Morphine. μOR has been shown to interact with δOR affecting its signaling properties.[8] To determine whether μOR−δOR interactions may be affected by morphine, we monitored changes in their association by FRET in Neuro-2a cells. In the absence of morphine, the degree of FRET between eYFP-μOR and eCFP-δOR is the same within error of eCFP-μOR and eYFP-μOR (0.45 ± 0.03, $n = 8$) supporting the ability of the receptors to form oligomers. This similarity suggests that the formation of μOR-δOR heteromers is of the same order as μOR homomers.

We then determined whether chronic morphine treatment will affect the association between eYFP-μOR and eCFP-δOR. Unlike the eYFP-μOR/eCFP-μOR homomers, morphine causes a reduction in FRET (Figure 3A). Note that distributions of FRET values, in both the basal and treated states, are fairly large, reflecting a heterogeneous population of eCFP-δOR/eYFP-μOR heteromers. The observation that a substantial population of the receptors is still associated with morphine treatment suggests either that morphine is changing the conformation between eCFP-δOR and eYFP-μOR resulting in less Förster transfer or that it is promoting dissociation of the receptors from higher order complexes.

To determine whether the decrease in eYFP-δOR and eCFP-μOR FRET was caused by protein dissociation, we measured the mobility of eYFP-μOR when coexpressed with unlabeled δOR in Neuro-2a cells with morphine treatment. We found that in the presence of δOR, the diffusion of μOR slows from $7.3 \pm 0.2 \times 10^{-9}$ to $6.0 \pm 0.1 \times 10^{-9}$ cm^2/s suggesting an increase in the size of the protein complex. This change could correlate with a shift of the μOR population to higher order oligomers. Surprisingly, treatment of cells expressing δOR and eGFP-μOR with 1 μM morphine for 24 h significantly increased the rate of diffusion to 13.6×10^{-9} cm^2/s . This increase was dose-dependent showing smaller increases (34 and 32%) at intermediate (0.05 and 0.1 μM) morphine concentrations and a 90% increase at 0.5 μM morphine. Co-treatment with 1 μM of the antagonist naloxone blocked changes in diffusion to give values identical to those

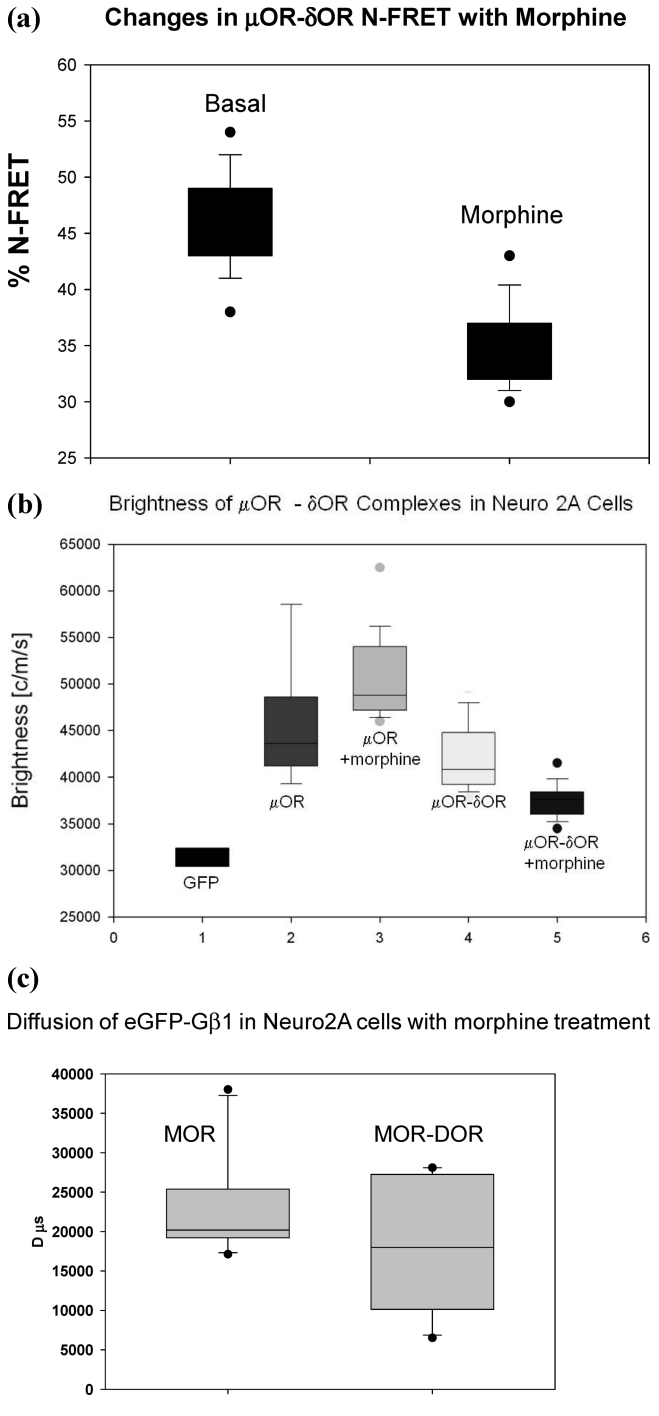

Figure 3. (A) Change in the normalized FRET between eYFP-μOR and eCFP-δOR with 1 μM morphine treatment in Neuro-2a cells. (B) Change in the molecular brightness in eYFP-μOR homomers and nonfluorescence δOR heteromers in Neuro-2a cells. (C) Change in the distribution of diffusion coefficients of eGFP-Gβ1 coexpressed with μOR-δOR in Neuro-2a cells with 1 μM morphine treatment for 48 h.

seen in the absence of morphine. Considering that the diffusion constant is inversely proportional to the square root of the mass, these measurements suggest that morphine disrupts larger complexes of μOR-δOR heteromers into smaller species.

Number and Brightness Analysis Suggests That δOR Promotes Morphine-Induced Dissociation of Receptor Complexes. To gain insight into the oligomerization state of the complexes, we determined the changes in number and brightness (N&B) of eGFP-μOR in Neuro-2a cells. N&B is based on the analysis of fluorescent fluctuation for each pixel in an image stack. The number of mobile particles and their brightness are obtained from the average intensity and the variance of the intensity in each pixel.[22] Nonfluorescent molecules are not observed, and immobile molecules produce a constant decrease in the intensity that is corrected in the analysis.

To establish the oligomerization of eGFP-μOR, we first measured free eGFP in Neuro-2a cells. This probe yielded a brightness of 30,000 c/m/s^2, which is routinely obtained in our experimental setup as well as those of others.[22] However, when we measure the brightness of an eGFP dimer, a value of 46,000 c/m/s^2 is obtained. This value is substantially less than the expected value of 60,000 c/m/s^2 for a protein dimer. To determine the reason for the reduced brightness of the eGFP dimer, we prepared cytosolic fractions of HEK293 cells expressing either single eGFP or double eGFP. We find that the anisotropy and emission spectrum of the two probes are identical, suggesting that energy transfer between the eGFP molecules in the double construct does not occur.[34] However, we also find that the quantum yield of the double eGFP is ~38% lower than the single mutant, implying that the reduced brightness of the double construct is due to self-quenching of the closely packed eGFP fluorophores. Since the receptors are all labeled on the C-terminus, then we would expect their labels to be similarly close in receptor dimers and obtain a brightness value close to 46,000 c/m/s^2.

For eGFP-μOR in Neuro-2a cells, we obtain a brightness of 45,000 c/m/s^2 which we interpret as corresponding to receptor dimers. Treatment of the cells with morphine shifts a portion of the population to higher values which may correspond to tetramers (Figure 3B) or a large separation between the subunits resulting in dequenching of the eGFP molecules. Note that the population of receptors that experiences this shift is not large enough to result in a significant shift in the diffusion.

When eGFP-μOR is coexpressed with unlabeled δOR in Neuro-2a cells, the brightness is similar to eGFP-μOR alone (i.e., ~45,000 c/m/s^2). Taking into account that we concomitantly observe a slower diffusion, we interpret this value as resulting association of μOR dimers with at least 2 δOR monomers to achieve units that have the brightness of the eGFP-μOR dimers with a slower diffusion. The simplest model is the formation of μOR-δOR tetramers. We note that the distribution of brightness of the complexes is broad suggesting that they contain 1−3 with an average of 2 μOR subunits.

Surprisingly, when cells expressing eGFP-μOR and δOR are treated with morphine, we find that the brightness of the eGFP-μOR/δOR complexes is substantially reduced to a value that corresponds to approximately a 50−50 mixture of eGFP-μOR-δOR dimers and eGFP-μOR dimers. This apparent dissociation of the mixed receptor complexes with morphine correlates well with the observed increase in mobility as measured by FCS (see above).

G-Protein Subunits May Detach from Larger Complexes with Morphine Treatment. Both our diffusion and brightness measurements show that μOR-δOR complexes dissociate with morphine treatment. Since morphine desensitization may involve decoupling of the receptors with G-proteins, we determined whether dissociation of receptor heteromers is accompanied by a change in receptor interactions with G-protein subunits. This study was carried out by measuring the change in diffusion with morphine treatment of eGFP-Gβ1 in Neuro-2a cells when cotransfected with either unlabeled μOR alone or with

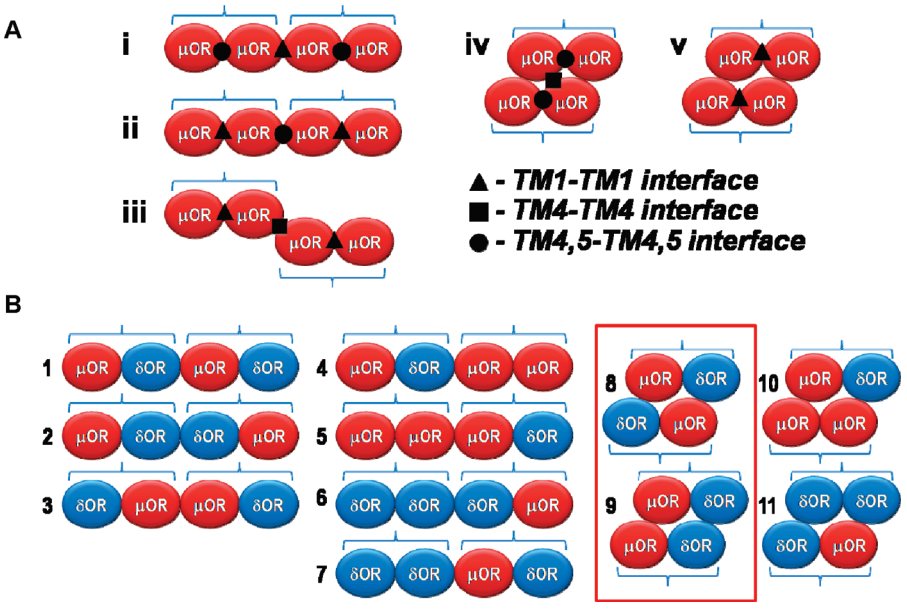

Figure 4. Possible configurations of μOR-μOR and μOR-δOR tetramers. (A) Cartoon of the possible permutations for the μOR homomeric tetramer. The compact (iv−v) and more extended (i−iii) arrangements differ in the combination of alternative dimeric interfaces within the tetramer: TM1-TM1 (▲), TM4-TM4 (■), and TM4,5-TM4,5 (●). (B) Cartoon of the possible permutations for the heteromeric tetramer formed by μOR (red oval) and δOR (blue oval). Depending on the different combinations of symmetric interfaces (2 for compact configurations and 3 for more extended ones) within the tetrameric arrangement, the total number of heteromeric permutations is 29 ($4 \times 2 = 8$ compact and $7 \times 3 = 21$ extended configurations). Permutations *8* and *9* (boxed in the figure) are the most likely candidates for μOR-δOR complexes based on consistency with the observed μOR-μOR and μOR-δOR FRET.

μOR and δOR. As expected, eGFP-Gβ1 has mobility similar to that of eGFP-OR when only μOR is expressed. However, when Neuro-2a cells are transfected with both μOR and δOR, a significant population of eGFP-Gβ1 shows increased mobility (Figure 3C). Keeping in mind that the transfection efficiency of the receptors is ∼40%, only ∼16% of eGFP-Gβ1 would be in cells containing both receptors. The results in Figure 3C suggest that the population of Gβ1 whose mobility increases is localized in cells expressing both receptors. Thus, even though morphine may induce dissociation of a significant population of μOR-δOR tetramers, interactions with G-proteins are maintained.

Structural Interpretation of Fluorescence Data. Since μOR and δOR are so closely related, the varying responses of the homo- and heteromers to morphine were unexpected. We thus took a molecular modeling approach to interpret the fluorescence data in structural terms. The simplest model that best fit the diffusion and brightness data is that the majority of μOR is dimeric and associates with δOR to form mixed tetramers. We first generated cartoon permutations of tetrameric μOR-μOR and μOR-δOR complexes (Figure 4), on the basis of the assumption that dimeric interfaces of opioid receptors involve preferentially TM1, TM4, and/or TM5, as is the case for several GPCRs.[27] In agreement with most available data, only symmetric TM1-TM1, TM4-TM4, or TM4,5-TM4,5 interfaces (represented in Figure 4A by filled triangles, squares, and circles, respectively) were considered. Figure 4A shows possible homomeric arrangements of μOR in a compact (permutations iv−v) or more extended (permutations i−iii) configuration. These five arrangements differ in the combination of possible symmetric interfaces within the μOR-μOR tetramer. However, at least 11 alternative symmetric permutations (Figure 4B) could be considered for μOR-δOR heteromeric tetramers. Depending on the different combinations of symmetric interfaces (2 for each compact configuration, and 3 for each of the more extended ones) within the tetrameric arrangement, the total number of heteromeric permutations is 29 (8 for compact configurations and 21 for more extended ones). Since our brightness data correspond to complexes that contain 2 eGFP-μOR, we can eliminate heteromeric permutations *4*−*7*, *10*, and *11* of Figure 4B. Keeping in mind that FRET has a steep distance dependence ($1/R^6$ where R is the distance between the fluorophores) and that the R_o (distance at which 50% of donor intensity is lost to transfer) is 50 Å for the eCFP/eYFP FRET pair,[35] we can eliminate permutations *1* and *2* of Figure 4B since the distance between the μOR subunits (i.e., 72 and 145 Å, respectively) would not give significant amounts of FRET. Permutation *3* of Figure 4B can also be eliminated because this model would give different FRET values for eCFP-μOR/eYFP-μOR versus eCFP-μOR/eYFP-δOR, and this is not observed. Thus, permutations *8* and *9* (boxed in Figure 4B) are proposed to be the most likely candidates for μOR-δOR complexes. Not only would these configurations be consistent with the observed μOR-μOR and μOR-δOR FRET, they are also consistent with the observation that the complexes contain two μOR subunits that are close enough to allow self-quenching. Thus, we generated energy-minimized 3D molecular models (Figure 5) of cartoon configurations *8* and *9* for μOR-δOR heteromeric complexes using the 2 possible combinations of interfaces in compact tetramers (see iv and v in Figure 4A), and the procedure described in Materials and Methods. A total of four μOR-δOR tetrameric models were built that either exhibited TM4,5-TM4,5 (Figure 5A and C) or TM1-TM1 (Figure 5B and D) heterodimeric interfaces. In these models, δOR was generated in an inactive conformation based on the inactive β2 adrenergic receptor crystal structure,[23] and μOR was built in an activated conformation based on the opsin crystal structure[25,26] (see

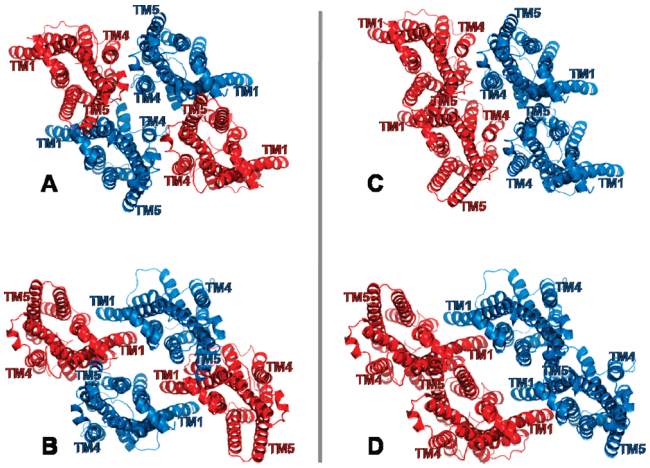

Figure 5. Energy minimized 3D molecular models of permutations *8* and *9* (from Figure 4B). (A and B) Models of permutation *8* exhibiting symmetric interaction of TM4,5 or TM1, respectively, at the heterodimeric μOR−δOR interface. (C and D) Models of permutation *9* exhibiting symmetric interaction of TM4,5 or TM1, respectively, at the heterodimeric μOR−δOR interface. In all cases, μOR is shown in red and δOR is shown in blue.

Materials and Methods). In Supporting Information, we present a cytoplasmic view of an overlap between the proposed active model of μOR (in red) and an inactive model of the receptor (in gray) based on opsin and the β2 adrenergic receptor crystal structures, respectively.

DISCUSSION

Determining the effects of prolonged morphine exposure on receptor organization might serve as a basis for understanding the mechanism of desensitization. The underlying cause of morphine tolerance is not understood but appears to culminate from a number of cellular effects. Several mechanisms of morphine desensitization have been proposed[1], and one of these involves uncoupling between μOR and the G-protein signaling cascade.[5] Here, we directly measured the ability of morphine to alter G-protein−receptor interactions using spectroscopic methods. Although we could not uncover evidence for μOR-G-protein uncoupling, we instead found that prolonged treatment with morphine alters the oligomerization behavior of opioid receptor heteromers. We carried out these studies by expressing fluorescent tagged proteins in neuronal cells and studied their association and movement. We first monitored the cellular localization of μOR and G-protein subunits. In accord with other studies, we find that these receptors are plasma membrane localized and remain on the membrane with morphine treatment. This result correlates well with biochemical and pharmacologic studies suggesting that desensitization is not due to a loss in receptor binding sites.[1]

We tested the idea that morphine treatment decreases the association between μOR and G-proteins by measuring the changes in FRET between eYFP-μOR and eCFP-Gβ1 in real time in living cells. We used Gβ1 for these studies since proteomic data from rat studies show that this protein is down-regulated with morphine addiction (Abul-Husn and Devi, unpublished work) and since Gβ subunits do not undergo the large structural changes seen in the activation of Gα which could affect the degree of FRET. We used eCFP-Gβ1γ7 since it has been established to have wild type cell properties.[15] We note that FRET values between eCFP-Gαi and eYFP-μOR were unchanged with continuous morphine treatment similar to the behavior seen for G$\beta\gamma$ and the receptor and that overexpression of Gαi did not affect the degree of FRET between eYFP-μOR and eCFP-Gβ1. Thus, our experiments show that G-proteins remain coupled to μOR with morphine binding in sharp contrast to the behavior seen for other receptor- G-protein systems.[36]

The R_0 for the CFP/YFP is 30 Å,[35] and thus a value of FRET greater than our negative controls will indicate that the proteins are physically associated when we consider the size of the proteins and the similar placement of the fluorescent tags. We note that the large uncertainty in probe orientation, as well as the potential contributions from multiple donors and acceptors, precludes us from drawing accurate distances from our FRET values. FRET studies between μOR and Gβ1 in two different neuronal cell lines suggest very minor changes with morphine treatment. This result argues against the idea that desensitization involves decoupling between receptor and G-proteins. Thus, morphine must induce other changes in the receptor that alter its ability to generate cell signals.

Since GPCRs may form oligomers, we focused on the ability of morphine to affect receptor−receptor interactions. Using single point FCS, we find that μOR displays a diffusion coefficient close to those reported for other GPCRs and small integral membrane proteins,[18] suggesting that the receptor is in the form of small oligomers. Addition of morphine does not significantly change this value. It is worthwhile to note that while previous FRAP and single point FCS studies show similar mobilities for GPCRs, different values can be found using other methods. For example, scanning FCS studies of a GFP-labeled bradykinin type 2 receptor (B2R) overexpressed in HEK293 cells can detect two additional populations with a 10-fold and 100-fold slower diffusion even though PCH analysis shows the receptor is diffusing as a homodimer.[18] Those results suggest maintenance of a dimeric form of this receptor even when localized in large complexes. In contrast, recent single molecule studies of muscarinic acid receptors in COS cells that followed the fluorescence from a bound ligand showed unrestricted diffusion of the monomeric and dimeric, or possibly dimeric to tetrameric forms of the receptor.[37] Similar to previous B2R studies,[18] our studies show that the brightness of μOR matches an eGFP dimer, suggesting a unit of two closely spaced μOR units. Addition of morphine results in a small increase in brightness without a significant change in mobility. This behavior is consistent with a shift from a predominantly dimeric population to a predominantly tetrameric population.

μOR is the primary mediator of morphine signals.[1] Since its oligomerization with δOR[8] is upregulated with chronic morphine,[11] we studied the interaction between these receptors using fluorescence methods in living cells. μOR and δOR were transfected under identical conditions, and visual inspection of transfected cells indicate that the two receptors are similarly expressed. It is important to note that in our experiments, we viewed the receptors under overexpressed conditions which may promote and stabilize oligomers. Although this behavior is not observed (see below), we predict that endogenous receptors might have a higher tendency to dissociate under natural conditions due to their lower concentrations.

Our FRET measurements show that in the basal state, μOR appears to have the same propensity to bind to δOR as it does to itself, suggesting that both homo- and heteromers form. Interestingly, morphine appears to stabilize μOR-μOR tetramers but destabilize μOR-δOR tetramers even though our measurements suggest that G-proteins remain associated with the receptors in

the associated or dissociated states. Rapid dissociation and reassociation of GPCR subunits in membranes has been reported,[37,38] and the reduced tendency of liganded-μOR tetramers to dissociate most likely reflects a stabilization of μOR contacts that increases its persistence time. To interpret the fluorescence data in a structural context, we first created cartoon permutations of μOR-μOR and μOR-δOR receptor tetramers containing activated μOR structures. On the basis of our fluorescence measurements, we were able to narrow down to four model structures the 29 different possibilities to assemble μOR and δOR involving the literature-predicted TM regions at symmetric heteromeric interfaces (e.g., TM1-TM1, TM4-TM4, and TM4,5-TM4,5). These four model structures differed in the presence of TM4,5-TM4,5 or TM1-TM1 at heteromeric interfaces. Although more experiments must be carried out using a series of mutant constructs to discriminate among these structures, these 4 models serve as a basis for a more detailed study of the functional properties of morphine receptors.

It is unclear whether disruption of opioid receptors oligomers is linked to morphine sensitization. Previous studies have shown that chronic morphine treatment leads to increased cell surface expression of μOR-δOR heteromers[39] and that dimerization with δOR leads to changes in the spatiotemporal dynamics of μOR signaling.[5] This behavior was mediated primarily by constitutive recruitment of arrestins to the heteromer. The fact that in this study we find association of Gβ1 with the heteromer under chronic morphine treatment suggests that the heteromer represents a multipart signaling complex that allows for the activation ofheteromer-specific, distinct signaling pathway. This model could, at least in part, contribute to μOR desensitization seen during the chronic exposure to morphine.

Our studies showing that morphine treatment does not perturb μOR subunit interactions or μOR−G-protein interactions, but destabilization of μOR-δOR oligomers can be interpreted in light of a recent study showing that activation of δOR in the heteromer leads to μOR degradation rather than recycling in turn diminishing its cellular response.[12] One can speculate that the smaller μOR-δOR oligomers can transfer into the lysozomal pathway more easily than larger μOR homomers through more accessible sites for proteolysis or modifications, such as ubitquitination. It is thus possible that the μOR-δOR system might be one of the prime examples of how the oligomerization state of a GPCR can mediate very different cellular responses.

■ ASSOCIATED CONTENT

Supporting Information. Cytosolic view of a homology model of active μOR superimposed on a model of the inactive form. This figure highlights the change in orientation of R165 and T279 when the receptor becomes activated. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

μOR, δOR, μ, δ opioid receptor; eCFP, eYFP, enhanced cynao or yellow fluorescent protein; FRET, Förster resonance energy transfer; FCS, fluorescence correlation spectroscopy; N&B, number and brightness.

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