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## Segregation of family A GPCR protomers in the plasma membrane

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## Abstract

G protein-coupled receptors (GPCRs) transduce many important physiological signals, and are targets for a large fraction of therapeutic drugs. Members of the largest family of GPCRs (family A) are thought to self-associate as dimers and higher-order oligomers, although the significance of such quaternary structures for signaling or receptor trafficking is known for only a few examples. One outstanding question is the physical stability of family A oligomers in cell membranes. Stable oligomers would be expected to move through cellular compartments and membrane domains as intact groups of protomers. Here we test this prediction by recruiting subsets of affinity-tagged family A protomers into artificial microdomains on the surface of living cells, and asking if untagged protomers move into these domains (are corecruited) at the same time. We find that tagged  $\beta_2$  adrenergic and  $\mu$  opioid protomers are unable to corecruit untagged protomers into microdomains. In contrast, tagged metabotropic glutamate receptor protomers do corecruit untagged protomers into such microdomains, which is consistent with the known covalent mechanism whereby these family C receptors dimerize. These observations suggest that interactions between these family A protomers are too weak to directly influence subcellular location, and that mechanisms that move these receptors between subcellular compartments and domains must operate on individual protomers.

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## Introduction

The largest family of transmembrane receptors is the G protein-coupled receptors (GPCRs; also known as seven-transmembrane receptors, 7TMRs), which includes several hundred individual gene products (Pierce et al., 2002). Recent studies have determined the tertiary structures of many of these receptors, and have uncovered conserved structural mechanisms of ligand binding, receptor activation, and G protein coupling (Kobilka, 2011; Rasmussen et al., 2011b; Rosenbaum et al., 2009). The quaternary structure of these receptors has also been studied extensively, as many GPCRs have been shown to associate as homodimers, heterodimers, or higher-order oligomers (Angers et al., 2002; Milligan, 2008; Palczewski, 2010). However, these studies have not revealed conserved structural mechanisms or functional consequences of oligomerization for the largest subset of GPCRs, the family A (rhodopsin-like) receptors. Individual receptor protomers are capable of binding ligands and coupling to downstream signaling molecules (Bayburt et al., 2011; Hanson et al., 2007; Whorton et al., 2007; Whorton et al., 2008). Therefore, the impact of family A receptor oligomerization may be restricted to allosteric regulation of receptor function (Han et al., 2009), or regulation of receptor trafficking and localization mediated by lateral interactions between protomers (Milligan, 2010). Methodological limitations have hindered the physical characterization of GPCR oligomers, and the stability and specificity of interactions between most family A protomers remain unknown or are controversial.

It is notoriously difficult to study interactions between integral membrane proteins *in vitro*, largely because it is difficult to isolate and solubilize intact proteins. Moreover, solubilization removes 2-dimensional constraints on membrane proteins, removes lipid components that may contribute to their structure, and alters the availability of other membrane proteins that may compete for or modify interactions. Therefore, interactions between isolated membrane proteins may differ substantially from those that occur in intact cellular membranes (Hong and Bowie, 2011). Resonance energy transfer (FRET and BRET) can be used to infer direct or indirect interactions between labeled membrane proteins in intact cells (Loura and Prieto, 2011; Milligan and Bouvier, 2005). However, distinguishing energy transfer due to a specific interaction from that due to random proximity is especially challenging when both labeled proteins are associated with a lipid bilayer (Vogel et al., 2006). Energy transfer between the same family A GPCRs has been attributed to specific association by some (Bouvier et al., 2007; Mercier et al., 2002) and random proximity by others (James et al., 2006), highlighting the need for additional methods to study interactions between transmembrane proteins in cells.

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Here we use micron-sized beads to recruit affinity-tagged class A protomers into microscopic domains on the surface of living cells while monitoring corecruitment of untagged protomers. This assay is conceptually similar to *in vitro* assays such as coimmunoprecipitation, but does not involve removal of receptors from the plasma membrane. We focus on  $\beta_2$  adrenergic receptors ( $\beta_2$ ARs), because these are prototypical family A receptors whose oligomerization has been studied in detail (Hebert et al., 1996). We also study  $\mu$  opioid receptors ( $\mu$ ORs), as these receptors crystallized with two large interprotomer interfaces that could mediate oligomerization in membranes (Manglik et al., 2012). We find that lateral interactions between protomers of these family A receptors are too weak to mediate corecruitment to bead-induced domains. This implies that oligomerization will also be unable to mediate significant corecruitment of these protomers into clathrin-coated pits or other cell surface domains.

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## Materials and Methods

**Plasmid DNA constructs.** SNAP- $\beta_2$ AR was purchased from New England Biolabs (Ipswich, MA); mGlu2 and  $\beta_2$ AR plasmids were purchased from the Missouri S & T cDNA Resource Center (Rolla, MO). Hexahistidine or the SNAP tag (preceded by the signal sequence from the 5HT3 receptor) was fused to the N-terminus, and cerulean or venus were fused to the C-terminus of mGlu2 and  $\beta_2$ AR. Hexahistidine-cerulean or venus (preceded by the signal sequence from the human growth hormone) were fused to the N-terminus of the rat  $\mu$ OR. The membrane marker RFP-mem was TagRFP fused to the final 25 amino acids of H-Ras. All constructs were made using a 2-step PCR protocol, and were verified by automated sequencing.

**Cell culture, transfection and labeling.** HEK 293, CHO-K1 and COS-7 (ATCC) cells were propagated in plastic flasks according to the supplier's protocol. Cells were split onto polylysine-coated glass coverslips 24-72 hours prior to transfection. Cells were transfected using 25kDa linear PEI (Polysciences, Inc., Warrington, PA) at an N/P ratio of 20, and were used for experiments 12-48 hours later. In most experiments the ratio of plasmid DNA encoding tagged receptor protomers to that encoding untagged protomers was 1:1. In order to estimate levels of receptor expression we measured  $\beta_2$ AR-V fluorescence intensity in populations of transiently-transfected cells using a fluorometer, and calculated receptor expression from a standard curve constructed using radioligand binding and a stable cell line that expressed  $\beta_2$ AR-V under the control of an inducible promoter. Correction for 30% transfection efficiency (measured using flow cytometry) yielded an average value of  $\sim 195,000$   $\beta_2$ AR-V receptor protomers per cell, or  $\sim 290 \mu\text{m}^{-1}$ . Judging from fluorescence intensity individual cells expressed a similar number of His- $\beta_2$ AR-C protomers. Cells expressing SNAP-tagged protomers were labeled with benzylguanine (BG) SNAP substrates in complete growth medium for 2 hours prior to imaging. For the experiment shown in Figure 5 cells were labeled with a mixture of 100 nM BG-649-PEG-biotin (New England Biolabs) and 5 nM SNAP-green (Cisbio, Codolet, France). The percentage of protomers labeled with each of these dyes was calculated by comparing green fluorescence emitted by cells labeled with this combination to that emitted by cells labeled with 100 nM SNAP-green alone using a plate-reading fluorometer. This concentration of SNAP-green saturates labeling of competent SNAP tags.

**Bead application and confocal imaging.** IMAC and sAV beads (Dynabeads His-Tag and sAV C1; Life Technologies, Carlsbad, CA) were washed in PBS, resuspended without dilution, and manually pipetted onto cells (10  $\mu\text{l}$  per 25 mm coverslip). According to the

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information supplied by the manufacturer (Dyna), streptavidin beads can bind  $\sim 400 \times 10^{12}$  moles of biotin per mg, which equates to approximately 80,000 molecules per  $\mu\text{m}^2$  of bead surface. Assuming that IMAC beads and streptavidin beads are similar ( $\sim 10^9$  beads  $\text{mg}^{-1}$ ), the corresponding value for IMAC beads would be  $\sim 275,000$  molecules  $\mu\text{m}^{-2}$ . Both of these values exceed the maximum possible packing density of 7TM receptors. Given the levels of receptor expression likely to be achieved in our experiments, the degree of recruitment observed (2-3 fold; e.g. Fig. S5), and the absence of bead saturation, the density of recruited protomers in our experiments is likely to be far less than these figures.

For imaging cells were bathed in PBS and imaged at room temperature (20-22°C) using a Leica (Wetzlar, Germany) SP2 laser scanning confocal microscope and a 63X, 1.4 NA objective. Excitation/emission wavelengths for the various fluorophores were (nm): cerulean, 458/465-500; venus, 514/520-650; TagRFP, 543/550-625; BG-649-PEG-biotin, 633/640-800; SNAP-green, 488/495-550. Image analysis was performed with ImageJ. Recruitment and corecruitment indices were calculated from background-subtracted regions of interest as indicated in Figure 1. Numerical values throughout the text refer to the mean  $\pm$  S.E.M..

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## Results

In order to establish an on-cell corecruitment assay we fused a hexahistidine (His) tag to the N-terminus and a fluorescent protein (cerulean, C or venus, V) to the C-terminus of the human  $\beta_2$ AR. The N- and C-terminal extensions of this receptor are relatively long (~30 and 80 amino acids, respectively) and unstructured, and have been shown previously to tolerate such additions with minimal disruption of receptor function (Barak et al., 1997b). Confocal imaging showed that His-tagged  $\beta_2$ ARs (e.g. His- $\beta_2$ AR-V) were expressed uniformly at the plasma membrane of transiently-transfected HEK293 cells (Fig. 1B). We then applied a suspension of cobalt-based immobilized metal affinity chromatography (IMAC) beads, which were allowed to settle onto the surface of transfected cells (Fig. 1A). After contacting the cell surface individual beads accumulated fluorescence over the course of ~1-2 minutes, which is consistent with diffusion to and capture of His-tagged protomers at the bead-cell interface (Supplemental Movie 1). In most cases the optical plane of section was parallel to the cell surface (Fig. 1A), thus hemispheric domains of His- $\beta_2$ AR-V appeared as fluorescent circles or crescents depending on whether the bead came to rest on the cell center (circles) or margin (crescents). Bead image profiles decreased in diameter as the image plane was moved towards the cell center, thus protomers were captured at the entire bead-membrane interface (Supplemental Fig. 1). Bead-induced domains were easily identifiable by their size (1  $\mu$ M in diameter), non-fluorescent interiors, and immobility relative to structures such as intracellular vesicles. In many cases fluorescence originating from tagged proteins in regions of the plasma membrane that were not associated with a bead was barely detectable above background, suggesting that in these cases almost all of the His-tagged proteins were recruited to a bead-induced domain. Because beads deformed the plasma membrane it was necessary to normalize background-subtracted fluorescence intensity in bead-associated ( $I_{\text{bead}}$ ) and surrounding ( $I_{\text{no bead}}$ ) regions of interest to the amount of membrane present in the same regions using an inert marker of the plasma membrane (Fig. 1B). For this purpose we used tagRFP bearing the C-terminal 25 amino acids of H-Ras (RFP-mem), which is both prenylated and palmitoylated. Corrected recruitment ( $R$ ; for tagged protomers) and corecruitment ( $C$ ; for untagged protomers) indices were defined as the fold fluorescence enhancement at the bead compared to the surrounding plasma membrane. Values of  $R$  or  $C$  greater than 1 indicate recruitment or corecruitment, values less than 1 indicate exclusion, and values of 1 indicate neither.



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We then asked if receptors retained their ability to bind ligands and signal to intracellular proteins while tethered to beads. We first transfected cells with His-tagged  $\beta_2$ AR-cerulean (His- $\beta_2$ AR-C) and venus-arrestin3 (V-arrestin3), recruited His- $\beta_2$ AR-C to beads, and then stimulated these receptors with the agonist isoproterenol (10  $\mu$ M; Fig. 1C). Agonist stimulation rapidly redistributed V-arrestin3 from the cytosol to the plasma membrane (Barak et al., 1997a), and in particular to regions of the plasma membrane associated with beads (Fig. 1D; Supplemental Movie 2). We then transfected cells with His- $\beta_2$ AR-C and Nb80-GFP, an engineered biosensor that binds to the active conformation of the  $\beta_2$ AR (Irannejad et al., 2013; Rasmussen et al., 2011a). Agonist stimulation rapidly redistributed Nb80-GFP from the cytosol to regions of the plasma membrane associated with beads (Supplemental Fig. 2; Supplemental Movie 3). These results suggest that  $\beta_2$ ARs concentrated at cell surface domains by IMAC beads remain functional, and are consistent with a previous study that showed that isolated  $\beta_2$ ARs remained functional after tethering to a solid substrate via an N-terminal tag (Neumann et al., 2002).

We reasoned that if GPCRs formed sufficiently stable dimers (or higher-order oligomers) at the cell surface, then recruitment of His-tagged protomers to bead-induced domains would corecruit untagged protomers to the same domains (Fig. 2A). As a positive homodimeric control we used the channelrhodopsin chimera C1C2. This seven-transmembrane protein was chosen because it closely resembles family A GPCRs in overall structure and topology, but has a conserved and structurally well-defined mechanism of homodimerization mediated by 2 or 3 disulfide bonds in a short N-terminal extension (Kato et al., 2012). His-tagged C1C2-cerulean (His-C1C2-C) corecruited untagged C1C2-venus (C1C2-V) to IMAC beads ( $C=1.79 \pm 0.09$ , mean  $\pm$  S.E.M.;  $n=28$ ; Fig. 2B). In contrast, His- $\beta_2$ AR-C did not corecruit C1C2-V ( $C=0.97 \pm 0.05$ ;  $n=24$ ; Fig. 2B), suggesting that association of C1C2-V fluorescence with IMAC beads depended on dimerization with His-C1C2-C protomers. In a similar manner, bead-associated His-C1C2-C did not corecruit untagged  $\beta_2$ AR-V ( $C=1.05 \pm 0.09$ ;  $n=25$ ; Fig. 2D). Finally, we found that recruitment of His- $\beta_2$ AR-C did not corecruit  $\beta_2$ AR-V ( $C=0.96 \pm 0.02$ ;  $n=40$ ; Fig. 2C and D). Similar results for His- $\beta_2$ AR-C and  $\beta_2$ AR-V were obtained in the presence of isoproterenol (10  $\mu$ M;  $C=0.97 \pm 0.04$ ;  $n=10$ ) or the inverse agonist ICI 118,551 (10  $\mu$ M;  $C=1.12 \pm 0.07$ ;  $n=10$ ). Recruitment of His- $\beta_2$ AR-C also failed to corecruit  $\beta_2$ AR-V in CHO-K1 and COS-7 cell lines (Supplemental Fig. 3). These results suggest that C1C2, but not  $\beta_2$ AR protomers, assemble as stable dimers on the cell surface.

Because the recent crystal structure of the  $\mu$ OR included two parallel dimer interfaces (Manglik et al., 2012) we performed similar experiments with His-cerulean- $\mu$ OR and venus- $\mu$ OR

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(His-C- $\mu$ OR and V- $\mu$ OR), both of which carried N-terminal fluorescent proteins. We have previously shown that similar fluorescent protein- $\mu$ OR fusion proteins are functional when immobilized on the cell surface (Lober et al., 2006). Accordingly, agonist stimulation (10  $\mu$ M DAMGO) rapidly redistributed V-arrestin3 from the cytosol to the bead-associated plasma membrane in cells expressing His-C- $\mu$ OR (Supplemental Fig. 4). However, recruitment of His-C- $\mu$ OR to IMAC beads failed to corecruit V- $\mu$ OR ( $C=0.94 \pm 0.04$ ;  $n=23$ ; Supplemental Fig. 4), suggesting that these family A receptors also do not assemble as highly stable dimers or higher-order oligomers in the plasma membrane.

Because His-tagged protomers were highly concentrated at the bead-membrane interface in these experiments, we considered the possibility that untagged protomers were sterically excluded from the membrane apposed to the bead surface. However, in cases where corecruitment was not observed  $C$  remained close to 1, indicating that untagged protomers were as abundant in regions of the plasma membrane associated with beads as in the surrounding regions. Exclusion of untagged protomers from bead-associated membrane regions would be expected to produce values of  $C$  that are less than 1. In addition, the nearly complete depletion of His-tagged protomers from non-bead membrane regions (e.g. Fig. 2B) suggested that bead binding capacity was not saturated, and that additional protomers could therefore have been accommodated in the membrane opposite the bead surface. To directly test this idea we cotransfected His- $\beta_2$ AR-C with either  $\beta_2$ AR-V (as before) or His- $\beta_2$ AR-V and imaged cells with standardized illumination and detection parameters. The absolute intensity of bead-associated His- $\beta_2$ AR-C was the same with  $\beta_2$ AR-V (125  $\pm$  9 a.u.;  $n=10$ ) and His- $\beta_2$ AR-V (131  $\pm$  7 a.u.;  $n=13$ ;  $P=0.62$ , unpaired t-test), suggesting that His-tagged protomers did not compete for limited binding sites or space in the bead-apposed membrane. Untagged  $\beta_2$ AR-V was not corecruited in these experiments ( $C=0.97 \pm 0.04$ ), whereas His- $\beta_2$ AR-V was heavily recruited ( $R=2.32 \pm 0.20$ ; Supplemental Fig. 5). Finally, we observed that  $\beta_2$ AR-V fluorescence remained the same in bead-apposed and surrounding regions during the process of recruitment, that is when His- $\beta_2$ AR-C was still in the process of being captured by the beads (Supplemental Fig. 6; Supplemental Movie 4). Disruption of dimers by steric exclusion would be expected to occur only as beads reached their full binding capacity, thus the absence of corecruitment at early time points is unlikely to be due to such a mechanism.

Unlike family A receptors, family C GPCRs dimerize via well-understood mechanisms, and dimerization has obvious functional consequences for these receptors (Pin et al., 2003). We were unable to demonstrate dimerization of the class C metabotropic glutamate (mGlu2) or

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GABA (GABA(B)) receptors with our original corecruitment strategy because we found that both receptors, unlike the family A receptors we tested, were nonspecifically recruited to IMAC beads even when they were not tagged with hexahistidine (Supplemental Fig. 7). This prompted us to explore other bead matrices and affinity tags, including biotin-avidin. In order to specifically biotinylate cell surface receptors we made use of the alkyltransferase SNAP-tag (Keppler et al., 2003), which has been used in several previous studies of GPCR oligomerization. In these experiments protomers were fused to either the SNAP-tag at their N-terminus and to cerulean at their C-terminus, or solely to venus at their C-terminus (Fig. 3A). SNAP-tagged protomers were covalently biotinylated with a biotin substrate (SNAP-biotin) and recruited to streptavidin (sAV)-conjugated beads. For example, biotinylated SNAP-mGlu2-cerulean (SN-mGlu2-C) corecruited mGlu2-venus (mGlu2-V) to sAV beads ( $C=1.54 \pm 0.07$ ,  $n=27$ ; Fig. 3 B and D). In contrast, biotinylated SN-mGlu2-C did not corecruit  $\beta_2$ AR-V ( $C=0.88 \pm 0.04$ ;  $n=17$ ), indicating the specificity of the mGlu2-V corecruitment. Conversely, when biotinylated SNAP- $\beta_2$ AR-cerulean (SN- $\beta_2$ AR-C) was recruited to sAV beads, neither mGlu2-V ( $C=0.87 \pm 0.06$ ;  $n=19$ ) nor  $\beta_2$ AR-V ( $C=0.96 \pm 0.03$ ;  $n=17$ ; Fig. 3 C and D) was corecruited. These results demonstrate the utility of this general strategy for GPCRs, and further suggest that  $\beta_2$ ARs are not held tightly together on the cell surface.

The availability of two different recruitment methods allowed us to simultaneously recruit different protomers to different cell surface domains. We transfected cells with His- $\beta_2$ AR-C and SN- $\beta_2$ AR-V, biotinylated the latter, and applied a mixture of IMAC and sAV beads. This resulted in the accumulation of cerulean and venus fluorescence at different regions of interest (ROIs) corresponding to IMAC beads and sAV beads (Fig. 4A; Supplemental Movie 5). Analysis of these regions over time indicated that IMAC beads (cyan ROIs) did not accumulate venus fluorescence, and sAV beads (yellow ROIs) did not accumulate cerulean fluorescence at any point during the recruitment process (Fig. 4B).

It is conceivable that only identical protomers assemble into stable homodimers, particularly if, as has been suggested, dimers form shortly after protein translation in the endoplasmic reticulum (Salahpour et al., 2004). In this case we would not expect corecruitment of protomers bearing different genetically-encoded tags and fluorescent proteins. In order to address this possibility we exploited the versatility of the SNAP-tag to perform corecruitment experiments using a protocol that required expression of only a single type of protomer. We transfected cells with either SN-mGlu2 or SN- $\beta_2$ AR (without C-terminal fluorescent proteins), and simultaneously labeled these receptors with a combination of spectrally-distinct SNAP substrates. One substrate (BG-649-PEG-biotin; Supplemental Fig. 8) included a biotin moiety

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and an infrared dye, whereas the other substrate (SNAP-green) was simply a green dye (Fig. 5A). The labeling ratio was adjusted so that ~60% of the protomers were labeled with BG-649-PEG-biotin, ensuring that a sufficient fraction of any dimers formed would bear both labels. When SN-mGlu2 protomers labeled with BG-649-PEG-biotin were recruited to sAV beads, SN-mGlu2 protomers labeled with SNAP-green were corecruited ( $C=2.08 \pm 0.25$ ;  $n=8$ ; Fig. 5 B and D). In contrast, when SN- $\beta_2$ AR protomers labeled with BG-649-PEG-biotin were recruited to sAV beads, SN- $\beta_2$ AR protomers labeled with SNAP-green were not corecruited ( $C=0.89 \pm 0.04$ ;  $n=9$ ; Fig. 5 B and D). These results suggest that the absence of  $\beta_2$ AR corecruitment cannot be explained by obligatory homodimerization of identical protomers.

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## Discussion

The idea that family A GPCR protomers interact with each other is now firmly established, but many questions concerning the functional significance and physical properties of these interactions remain unanswered. Here we address the physical stability of interactions between  $\beta_2$ AR and  $\mu$ OR protomers using a quantitative imaging approach in living cells. We find that recruiting a subset of protomers into artificial cell surface domains does not lead to detectable indirect corecruitment of other protomers, suggesting that the interactions between protomers are not sufficiently stable to dictate residence in such domains. Measuring steady-state corecruitment of untethered protomers to domains of tethered protomers does not yield an association lifetime, but rather indicates the state of the association-dissociation equilibrium. Our results suggest that, for the receptors we studied, this equilibrium greatly favors dissociation. This appears to be the case even though the receptors we studied were overexpressed, and were likely to be more concentrated than receptors expressed in most native tissues. We cannot rule out the possibility that non-covalent dimers are in some way disrupted when one protomer of a pair binds to a bead. This type of disruption would have to apply to three types of N-terminal affinity tags (His-, His-cerulean- and SNAP-) and two types of beads (IMAC and streptavidin) in order to completely account for our failure to observe corecruitment of untagged  $\beta_2$ AR and  $\mu$ OR. Moreover, we have shown that bead-attached protomers are functionally intact to the extent that they still bind agonists and undergo the conformational changes associated with signaling. This suggests that tethering to a solid substrate via an N-terminal tag does not substantially alter receptor structure or dynamics, and therefore it seems unlikely that it would disrupt dimerization.

The absence of corecruitment into bead-induced domains is consistent with our previous observation that internalization of agonist-bound  $\beta_2$ AR protomers does not lead to significant cointernalization of unbound protomers (Lan et al., 2011), and is also consistent with our previous fluorescence recovery after photobleaching (FRAP) study of D2 dopamine receptors, which indicated that protomers of these monoamine receptors do not detectably influence one another's mobility in the membrane (Fonseca and Lambert, 2009). On the other hand, other studies using FRAP and single-molecule imaging methods have concluded that  $\beta_2$ ARs form relatively stable (~5 second lifetime) higher-order oligomers, and that the equilibrium favors association even with low (subphysiological) levels of receptor expression (Calebiro et al., 2013; Dorsch et al., 2009). The reason(s) for these discrepant results are not immediately apparent. Similar unresolved discrepancies exist between different FRET and BRET studies of  $\beta_2$ AR

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oligomerization (Felce and Davis, 2012; James et al., 2006; Kawano et al., 2013; Mercier et al., 2002; Salahpour and Masri, 2007).

The physiological function of GPCRs involves the regulated movement of receptors between subcellular compartments and retention of receptors in discrete domains. Our findings suggest that the mechanisms that direct the family A receptors we studied into subcellular compartments must operate on individual protomers, as opposed to dimers or oligomers. Like many GPCRs, these receptors are recruited into clathrin-coated pits by binding to arrestin, which in turn interacts with clathrin and the clathrin adapter AP2 (Goodman et al., 1996; Kang et al., 2013). Our results suggest that each protomer will need to bind directly to an arrestin molecule in order to be recruited into a coated pit. In a similar manner,  $\beta_2$ ARs are not distributed randomly on the surface of cardiomyocytes, but are confined in plasma membrane microdomains via an interaction between the receptor C-terminus and an A-kinase anchoring protein (AKAP) (Valentine and Haggie, 2011). Our results suggest that each protomer will be retained separately by this mechanism. Likewise, postendocytic sorting of  $\beta_2$ ARs and other GPCRs relies on interactions with adapter proteins and elements of the cytoskeleton (Cao et al., 1999; Hanyaloglu and von Zastrow, 2008), and our results suggest that individual protomers, rather than groups of protomers, will be sorted by these mechanisms. It is quite possible that interactions between other family A protomers will be found to be much more stable than those that we studied here. However, at least for the receptors that we studied, our results suggest that the functional significance of oligomerization will not include localization in cellular compartments and domains.

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### **Authorship Contributions**

*Participated in research design:* Javitch and Lambert.

*Conducted experiments:* Gavalas, Lan and Lambert.

*Contributed new reagents:* Liu and Corrêa.

*Performed data analysis:* Gavalas, Lan and Lambert.

*Wrote or contributed to the writing of the manuscript:* Javitch and Lambert.

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### **Footnotes**

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## Legends for Figures

**Fig. 1.** IMAC beads recruit functional  $\beta_2$  adrenergic receptors. (A) Schematic illustrating recruitment of His- $\beta_2$ AR-V protomers to the surface of immobilized metal affinity chromatography (IMAC) beads. The dashed line indicates the typical optical plane of section. Intensity of RFP-mem in bead-associated ( $I_{\text{bead(mem)}}$ ) and surrounding ( $I_{\text{no bead(mem)}}$ ) regions of interest (ROIs) is used to correct for distortion of the plasma membrane by the bead and calculated recruitment ( $R$ ) and corecruitment ( $C$ ) indices. (B) Confocal images showing His- $\beta_2$ AR-V and RFP-mem before and after recruitment to IMAC beads, and illustration of regions of interest used for analysis and background (bg) subtraction; scale bar, 5  $\mu\text{m}$ . (C and D) Bead-associated His- $\beta_2$ AR-C recruits V-arrestin3 from the cytosol to the plasma membrane in response to 10  $\mu\text{M}$  isoproterenol; scale bar, 2  $\mu\text{m}$ . Data points in D represent the mean  $\pm$  S.E.M. of 5 cytosol and 27 bead ROIs from 5 cells.

**Fig. 2.** Corecruitment of channelrhodopsin (C1C2) but not  $\beta_2$ AR protomers to IMAC beads. (A) Stable dimers corecruit ( $C > 1$ ) untagged protomers to IMAC beads (*left*), whereas monomers leave untagged protomers distributed throughout the surrounding plasma membrane ( $C = 1$ ; *right*). (B and D) His-C1C2-C corecruits C1C2-V, but not  $\beta_2$ AR-V to bead-associated regions (*white arrowheads*). Plasma membrane regions not apposed to IMAC beads (*red arrowheads*) are severely depleted of His-C1C2-C protomers. (C and D) His- $\beta_2$ AR-C does not corecruit  $\beta_2$ AR-V to IMAC beads. Scale bars in B and C, 2  $\mu\text{m}$ . In D bars represent median, 25<sup>th</sup> and 75<sup>th</sup> percentiles, small squares represent mean values, and whiskers represent minimum and maximum values.

**Fig. 3.** Corecruitment of metabotropic glutamate (mGlu2) but not  $\beta_2$ AR protomers to streptavidin (sAV) beads. (A) SNAP-tagged (SN-) protomers are covalently labeled with BG-biotin and fused to cerulean, whereas protomers without SNAP tags are fused to venus. (B and D) Biotinylated SN-mGlu2-C corecruits mGlu2-V to sAV beads. (C and D) Biotinylated SN- $\beta_2$ AR-C does not corecruit  $\beta_2$ AR-V. Scale bars in B and C, 2  $\mu\text{m}$ .

**Fig. 4.** Segregation of His- $\beta_2$ AR-cerulean and SN- $\beta_2$ AR-venus by mixed IMAC and sAV beads. (A) Mixed IMAC and sAV beads are applied to cells expressing His- $\beta_2$ AR-C and SN- $\beta_2$ AR-V. Cyan and yellow ROIs are indicated; scale bar, 2  $\mu\text{m}$ . (B) Development of cyan and yellow

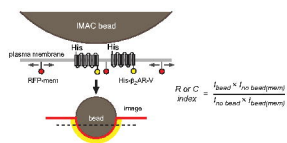
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fluorescence over time in ROIs designated cyan or yellow. Beads are pipetted over cells at time = 30 seconds, and settle onto the cell surface over the next minute. Individual data points represent the mean  $\pm$  S.D. of 15 cyan and 15 yellow ROIs from two cells.

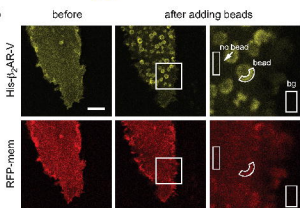
**Fig. 5.** Corecruitment of SNAP-mGlu2 but not SNAP- $\beta_2$ AR protomers labeled with dye (SNAP-green) by protomers labeled with dye-biotin (BG-649-PEG-biotin). (A) SNAP-tagged protomers are labeled with BG-649-PEG-biotin or SNAP-green after incubation with a mixture of both dyes. (B and D) SN-mGlu2 protomers labeled with BG-649-PEG-biotin corecruit protomers labeled with SNAP-green to sAV beads. (C and D) SN- $\beta_2$ AR protomers labeled with BG-649-PEG-biotin do not corecruit protomers labeled with SNAP-green. Scale bars in B and C, 5  $\mu$ m.

Figure 1

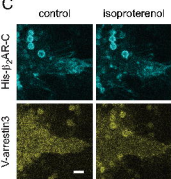
A



B



C



D

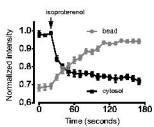


Figure 2

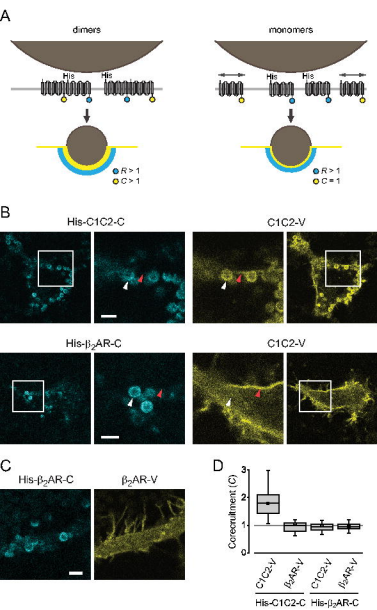




Figure 3

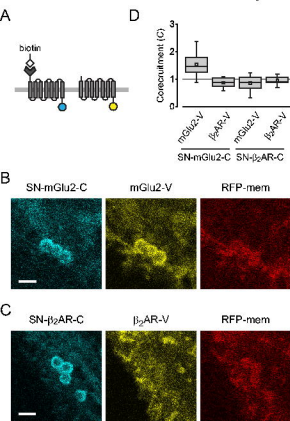
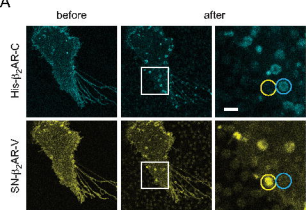


Figure 4

A



B

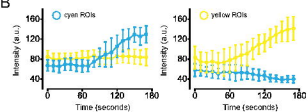
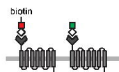
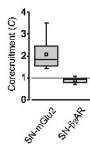


Figure 5

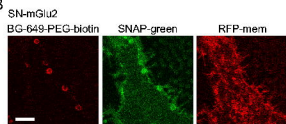
A



D



B



C

