GHSR-D2R heteromerization modulates dopamine signaling through an effect on G protein conformation

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The growth hormone secretagogue receptor (GHSR) and dopamine receptor (D2R) have been shown to oligomerize in hypothalamic neurons with a significant effect on dopamine signaling, but the molecular processes underlying this effect are still obscure. We used here the purified GHSR and D2R to establish that these two receptors assemble in a lipid environment as a tetrameric complex composed of two each of the receptors. This complex further recruits G proteins to give rise to an assembly with only two G protein trimers bound to a receptor tetramer. We further demonstrate that receptor heteromerization directly impacts on dopamine-mediated Gi protein activation by modulating the conformation of its α-subunit. Indeed, association to the purified GHSR:D2R heteromer triggers a different active conformation of Gαi that is linked to a higher rate of GTP binding and a faster dissociation from the heteromeric receptor. This is an additional mechanism to expand the repertoire of GPCR signaling modulation that could have implications for the control of dopamine signaling in normal and physiopathological conditions.

GPCR | G protein | heteromer | conformational dynamics | signaling

G protein-coupled receptors (GPCRs) are membrane proteins involved in regulating virtually every aspect of physiology. Most membrane receptors assemble as oligomeric complexes to fulfill their function (1). GPCRs do not escape the rule, as homo- and hetero-oligomers have been described for many different receptors (2). Although monomers can activate G proteins and recruit arrestins (3–5), association of different GPCRs within the same assembly can give rise to complexes with distinct and unique properties (6). This heteromer-directed signaling process has been proposed to be pivotal in modulating some of the physiological function of GPCRs as well as in disease-associated deregulations of signaling (6).

Ghrelin is a peptide hormone that has emerged as a major gut–brain signal controlling, among others, growth hormone secretion, food intake, and reward-seeking behaviors (7). These effects result from its interaction with the growth hormone secretagogue receptor (GHSR), a typical peptide-activated class A GPCR (7). Multiple data indicate that GHSR dimerizes with a wide array of GPCRs and that heteromerization could have an impact on its signaling properties and on those of the associated receptors (8). In this context, dimerization of GHSR with the dopamine D2 receptor is of particular interest. Indeed, assembly of D2R with apo-GHSR, which has been demonstrated in heterologous systems and hypothalamic neuron cultures, modulates dopamine-dependent signaling through the βγ subunits of D2R-associated Gi (9). However, so far, the molecular processes responsible for the impact of GHSR on D2R pharmacology remain to be illuminated.

To disentangle the mechanism through which interaction of apo-GHSR with D2R could affect dopamine signaling, we assembled the purified ghrelin and dopamine receptors into lipid vesicles. By doing so, we demonstrate that, in liposomes, these two receptors interact to form a tetrameric complex comprising two each of GHSR and D2R. We further bring evidence that, in this model system, heteromerization alters dopamine-dependent Gi activation by modulating the conformational features of the $G\alpha_{i1}$ subunit.

Results

Hetero-Oligomeric Arrangement of the Ghrelin and Dopamine Receptors in Lipid Vesicles. We first investigated the propensity of GHSR to oligomerize when inserted in 200-nm PC/PE/PS/cholesterol large unilamellar vesicles. Large unilamellar vesicles were selected to limit segregation effects associated with membrane curvature (10). Receptor oligomerization was investigated using three-color FRET where fluorescein (Fluo), Cy3, and Cy5 were used as the donor, the transmitter, and the acceptor (11). To this end, isolated GHSR was labeled with either of the fluorophores on a unique reactive cysteine in the extracellular part of TM7 (C^{304}) (12), mixed in equimolar amounts, and inserted into the liposomes. After proteoliposome assembly, a significant signal was observed at the emission wavelength of both Cy3 and Cy5 upon

Significance

G protein-coupled receptors (GPCRs) are one of the largest cell surface receptor family that transmit their signal through coupling to intracellular partners, such as G proteins. Receptor oligomerization has been shown to be pivotal in this signaling process. To address how oligomerization can impact on signaling in a major physiological process, dopamine signaling, we used a purified GPCR heteromer composed of the ghrelin and dopamine receptors to which we applied a variety of state-of-the-art biochemical and biophysical approaches. By doing so, we provide a direct experimental evidence for a mechanism where receptor heteromerization affects the conformation of the associated G protein. This sheds light on the way a GPCR oligomer can affect G protein activation to modulate signaling.

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Fluo excitation (Fig. 1*A*). This points to an oligomeric assembly comprising at least three GHSR protomers.

We then assembled into the liposomes labeled GHSR with unlabeled potential partners and monitored through Fluo-Cy3 (two-color) and Fluo-Cy3-Cy5 (three-color) FRET whether the additional receptor competed with GHSR homo-oligomerization. Because GHSR is essentially monomeric before insertion into the liposomes (*SI Appendix*, Fig. S1), the assay reports on the propensity of isolated receptors to form oligomers upon detergent-to-lipid exchange. To validate the method, Fluo-, Cy3-, and Cy5-labeled GHSRs were first mixed with a 10-fold molar excess of unlabeled GHSR before assembly into liposomes. Under such conditions, a decrease of both two-color and three-color FRET signals was observed (Fig. 1*B*), indicating that the unlabeled receptor competed with the assembly of labeled-GHSR oligomers. Consistently, saturation-like plots were obtained when varying the molar ratio of unlabeled-to-labeled GHSR (Fig. 1*C*), with a percent of fluorescence change at a 1:1 molar ratio in the same range than that measured in a cellular system (9). We then investigated whether other purified GPCRs could compete with GHSR oligomer formation. These included two receptors that had been shown to dimerize with GHSR (D1R, D2R) and unrelated GPCRs [BLT1, BLT2, MT1R, 5-HT4(a)]. Either of these receptors was mixed with GHSR labeled with Fluo, Cy3, and Cy5 at a 10-fold GPCR-to-GHSR molar ratio and assembled into liposomes. A significantly lower FRET signal was observed in the presence of D1R and D2R only (Fig. 1*B*). In both cases, whereas a two-color FRET signal persisted, no more detectable three-color FRET could be observed. Of importance, most of the D2R and of the negative control receptors (BLT1, MT1R) inserted into the lipid vesicle in the same inside-in orientation as GHSR (*SI Appendix*, Fig. S2). A saturation-like profile was also obtained when the FRET signal was measured at increasing D2R-to-GHSR molar ratios (Fig. 1*C*). The ratio at which half of the change in the signal occurred was similar for two- and three-color FRET, suggesting that both changes result from the same molecular event. Overall, these data indicate that D2R disrupts GHSR oligomers while maintaining some homodimeric interactions. GHSR:D2R proximity in the lipid vesicles was further confirmed by measuring FRET between GHSR labeled with a Tb-cryptate donor on Cys^{304} and D2R labeled with an XL255-labeled antibody reacting with its N-terminal Flag-tag (Fig. 1*D*).

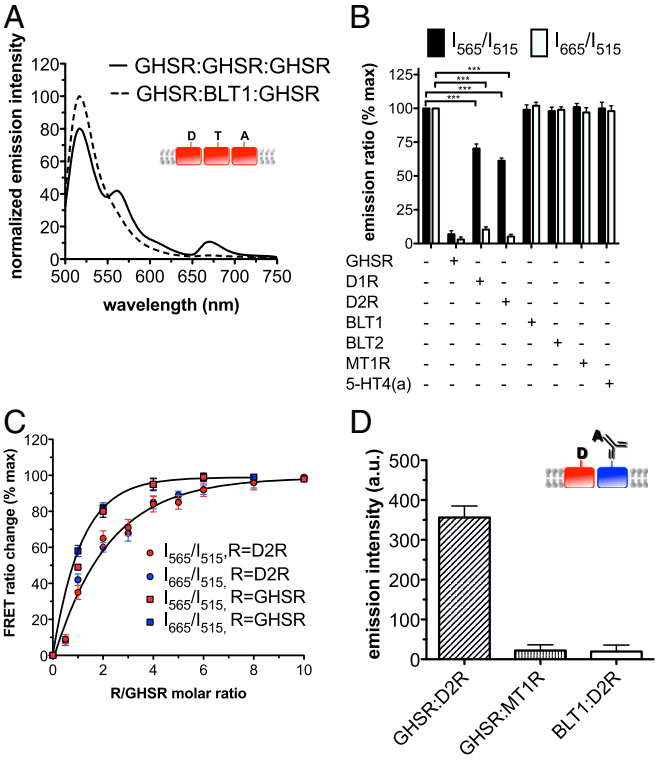

Fig. 1. GHSR:D2R oligomerization in proteoliposomes. (*A*) Fluorescence emission spectra (λ_{exc}: 495 nm) from proteoliposomes containing Fluo-GHSR, Cy3-GHSR, and Cy5-GHSR (plain lines) or Fluo-GHSR, Cy3-BLT1, and Cy5-GHSR (dotted lines). (*B*) Changes in Fluo-Cy3 (I_{565}/I_{515}) and Fluo-Cy3-Cy5 (I_{665}/I_{515}) FRET in proteoliposomes containing Fluo-, Cy3-, and Cy5-GHSR and a 10-fold higher molar ratio of unlabeled receptors. (*C*) Changes in normalized emission intensity of Cy3 (I_{565}/I_{515}) and Cy5 (I_{665}/I_{515}) as a function of the GHSR:GHSR (boxes) or GHSR:D2R (circles) molar ratio. Data are presented as the percent of maximal change in FRET signal observed at the 8:1 (GHSR:GHSR) or 10:1 (D2R:GHSR) ratio. (*D*) XL255 emission intensity after Tb-cryptate excitation of proteoliposomes containing Tb-cryptate–labeled GHSR and XL255-labeled D2R, Tb-cryptate GHSR, and XL255 MT1R, or Tb-cryptate BLT1 and XL255 D2R. The different species are schematically represented (red: GHSR, blue: D2R; D: Fluo donor, T: Cy3 transmitter, A: Cy5 acceptor). Data in *B* and *D* are the mean ± SD of three experiments (***$P < 0.001$).

The GHSR:D2R Heteromer. We then purified the GHSR:D2R complex from the proteoliposomes. To this end, lipid vesicles containing GHSR and D2R were solubilized with the styrene maleic acid (SMA) copolymer (13, 14) to get membrane discs (SMALPs) encapsulating the receptors. SMA solubilization of purified vesicles is a fast process (15) so that the complexes initially present in the proteoliposomes are likely to be trapped as such into the SMALPs. Particles in the 20-nm range were obtained after solubilization of the proteoliposomes with SMA at low polymer-to-lipid ratios and purification by tandem chromatography (*SI Appendix*, Fig. S3). The complex obtained under such conditions was functional with regard to ligand-binding, G protein activation, and arrestin recruitment (*SI Appendix*, Fig. S4), with both D2R and GHSR fully ligand-competent (*SI Appendix*, Fig. S5).

A significant two-color signal could be measured when the SMALPs were prepared from liposomes containing D2R and Fluo-, Cy3-, and Cy5-labeled GHSR, whereas no three-color FRET could be detected (Fig. 2*A*). This suggests the occurrence of two but no more GHSR protomers in the SMALPs. These SMALPs likely included more than a single receptor, at least in the case of GHSR, as suggested by a GHSR dimer purification assay (*SI Appendix*, Fig. S6). A significant homogenous time-resolved fluorescence (HTRF) signal could also be measured upon labeling with Tb- and XL255-labeled antibodies recognizing the Flag-tag of D2R (Fig. 2*A*), indicating the presence of at least two D2R protomers. Finally, the highest molecular-weight limit species after chemical cross-linking (16) had an electrophoretic mobility compatible with that of a tetramer composed of two D2R and two GHSR (Fig. 2*B* and *SI Appendix*, Fig. S7). Taken together, these data suggest that the major complex trapped from the proteoliposomes could be a tetramer composed of two each of GHSR and D2R.

We then considered the organization of the complex formed between the purified D2R:GHSR heteromer in SMALPs and G proteins. To this end, we labeled $G\alpha_i$ and $G\alpha_q$ at their N terminus with either a fluorescence donor (AF-350) or an acceptor (AF-488) (17) for G protein:G protein intermolecular FRET. $G\alpha_{i1}$ was used because GHSR does not couple to this G protein subtype in vitro (5) and in HEK cells (18). We first assembled the purified heteromer in SMALPs with AF350- and AF488-labeled $G\alpha_i\beta\gamma$ in equimolecular amounts. In the absence or presence of dopamine, no FRET signal could be observed (Fig. 2*C*). This was not a limiting effect of the receptor-to-G protein ratio used, as increasing this ratio did not induce any FRET signal. This suggests that the tetrameric complex recruits only one $G\alpha_i\beta\gamma$ trimer upon D2R activation. In the same way, no $G\alpha_q$:$G\alpha_q$ FRET could be detected in the absence or in the presence of ghrelin when incubating AF350- and AF488-labeled $G\alpha_q\beta\gamma$ with the D2R:GHSR heteromer (Fig. 2*C*). In contrast, a FRET signal was observed in the presence of dopamine with AF350-labeled $G\alpha_i$ and AF488-labeled $G\alpha_q$, whether ghrelin was present or not (Fig. 2*C*). A possibility would be to consider a model where both G

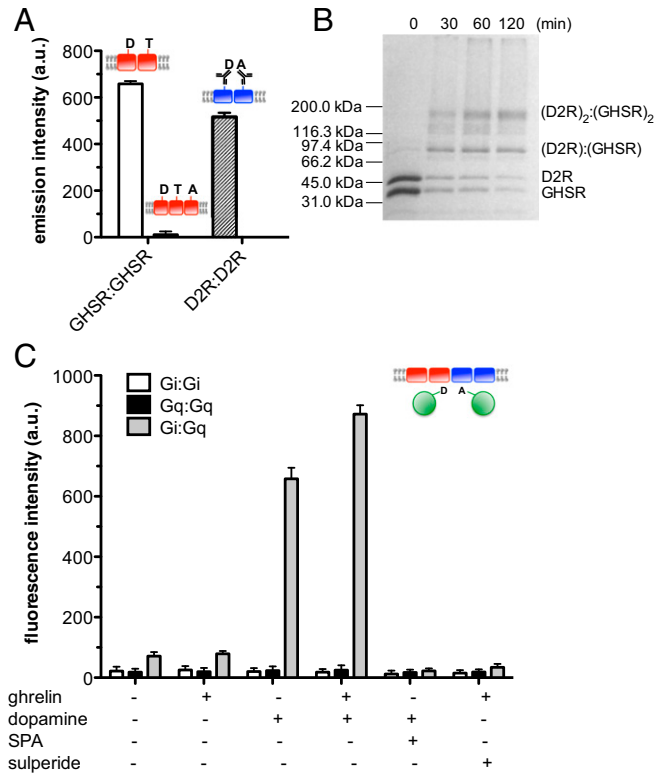

Fig. 2. Stoichiometry of the GHSR:D2R:G protein complex in SMALPs. (*A*) Two- and three-color GHSR:GHSR and Tb-cryptate:XL255 D2R:D2R FRET for heteromer-containing SMALPs. (*B*) SDS/PAGE monitored DMS cross-linking kinetics (0, 30, 60, and 120 min) of the heteromer in SMALPs. (*C*) FRET between G protein trimers where the Gα subunit was labeled with AF-350 and AF-488 in the presence of the GHSR:D2R heteromer in the absence or in the presence of ligands. The different species are schematically presented (red: GHSR; blue: D2R; green: G protein; A: acceptor; D: donor; T: transmitter). Data in *A* and *C* are the mean ± SD of three different experiments.

proteins would be associated to the receptor heterotetramer, with Gq preassembled with GHSR (17) and $G\alpha_i$ recruited to D2R upon dopamine-mediated activation (19). Accordingly, no FRET signal could be observed in the presence of the GHSR inverse agonist SPA that dissociates the preassembled GHSR:Gq complex (17) (Fig. 2*C*). Finally, a significant $G\alpha_i$:$G\alpha_q$ FRET signal was observed upon stimulation with both dopamine and ghrelin. This signal was slightly different from that in the absence of ghrelin (Fig. 2*C*), possibly because of a different arrangement of the preassembled and active GHSR:Gq complexes (17).

Impact of GHSR:D2R Association on G Protein Activation. We then analyzed whether heteromerization affected G protein activation. In this case, the model system included either the D2R homomer or the D2R:GHSR heteromer in SMALPs, $G\alpha_{i1}\beta\gamma$ and $G\alpha_q\beta\gamma$. The D2R homomer was obtained after solubilization of D2R-only proteoliposomes (*SI Appendix*, Fig. S8). We first investigated Gi activation by measuring the rate of association of GTPγS to $G\alpha_{i1}\beta\gamma$ in the preformed ternary complex. This rate was monitored through the changes in Trp emission that accompany GTPγS binding to $G\alpha_i$ (*SI Appendix*, Fig. S9). To avoid any interference from $G\alpha_q$, $G\alpha_{i1}$ was labeled with 5-hydroxytryptophan (5HW) to selectively excite it without affecting other Trp residues (20). The receptors in SMALPs were incubated in the absence or presence of dopamine with both G proteins, and the rate of GTPγS association to $G\alpha_i$ was measured. This rate was faster for the heteromer than for the homomer (Fig. 3*A* and *SI Appendix*, Table S1). The difference between the homomer and the heteromer was not affected by ghrelin but was no more observed in the presence of SPA or in the absence of Gq (Fig. 3 *B* and *C*).

We then investigated how G protein dissociated from the receptor after its activation. To this end, proteoliposomes were reconstituted in the presence of Fluo-labeled PE. SMALPs containing the fluorescent lipid and the different receptor protomers were then purified and assembled in the presence of dopamine with $G\alpha_q\beta_1\gamma_2$ and $G\alpha_i\beta_1\gamma_2$, with the βγ subunits of Gi only labeled with AF-350 (21). These subunits were selected for monitoring dissociation as, in cell-based assays, modulation of D2R signaling by GHSR involved βγ from Gi (9). To avoid anchoring of the G proteins to the lipid bilayer and the associated nonspecific FRET, we used the soluble unlipidated mutants of all G protein subunits (22). Accordingly, no major FRET signal was observed with empty SMALPs or with SMALPs containing only GHSR (*SI Appendix*, Fig. S10). We then monitored the changes in FRET between the G protein and the fluorescein-labeled SMALPs as a function of time after addition of GTPγS. This time-course was different, depending whether the D2R homomer or the D2R:GHSR heteromer were considered (Fig. 3*D*). While the difference was essentially not affected by ghrelin, it was abolished by SPA or in the absence of Gq (Fig. 3 *E* and *F*).

To then assess whether the effects we observed with the purified heteromer could impact on D2R signaling in a cellular environment, we monitored dopamine-mediated $G\alpha_{i1}$ activation in HEK293T/17 cells using the bioluminescence resonance energy transfer (BRET) signal between RLuc8-$G\alpha_{i1}$ and GFP2-$G\gamma_2$ (Fig. 3*G*). In this assay, dopamine promotes D2R activation and subsequent dissociation between Gα and Gβγ, thereby increasing the distance between the $G\alpha_{i1}$ energy donor and the $G\gamma_2$ energy acceptor and leading to a decrease in the BRET signal compared with the basal state (23–25). Hence, any effect of GHSR on D2R-dependent Gi activation should result in a change in the dopamine-modulated BRET signal. As shown in Fig. 3*H*, coexpressing GHSR with D2R decreased this BRET signal, indicative of an increase in Gi activation. This effect was dependent on the amount of GHSR and was not observed with an unrelated Gq-coupled GPCR, the purinergic receptor P2Y1 (Fig. 3 *H* and *I*). Of importance, cotransfecting the different receptors did not result in a significant variation in the level of $G\alpha_{i1}$ expression that could be responsible for the changes in BRET signal (*SI Appendix*, Fig. S11). These data suggest that apo-GHSR can increase dopamine-dependent $G\alpha_{i1}$ activation in a cellular context as well.

We finally investigated the conformational features of $G\alpha_i$ bound to homomer- and heteromer-containing SMALPs using intramolecular time-resolved luminescence resonance energy transfer (LRET) (26) with a lanthanide donor and a fluorescein acceptor both attached to $G\alpha_i$. To this end, we used $G\alpha_i$ mutants with two unique reactive cysteines at different positions (i.e., 90 and 238, 141 and 333, or 171 and 276) (27). LRET measurements were carried out by monitoring the decay in the acceptor-sensitized emission, so that only the donor and acceptor engaged in LRET were detected (26) for the doubly labeled $G\alpha_i$ of the $G\alpha_i\beta\gamma$ trimer in its free- and receptor-bound states (Fig. 4). The free-state corresponded to Gi in the presence of Gq and empty SMALPs, and the receptor-bound state to Gi in the presence of Gq, SMALPs containing either the D2R homomer or the D2R:GHSR heteromer, and saturating concentrations in dopamine. In all cases, measurements were carried out with the empty state of the G protein (no GTP added, 30-min apyrase treatment) and dopamine was preincubated with the receptor. All decay plots were best fitted by two exponentials with a short and a long component (*SI Appendix*, Table S2). The short component was always predominant in the absence of receptor, whereas the long one was the major component in the presence of the receptor. This suggests that $G\alpha_i$ is conformationally dynamic with at least two states whose relative abundance is governed by its coupling to the receptor. Whereas the short component was essentially unchanged, the long one was different depending whether the G protein was associated to the dopamine-activated homomer or heteromer. The major difference

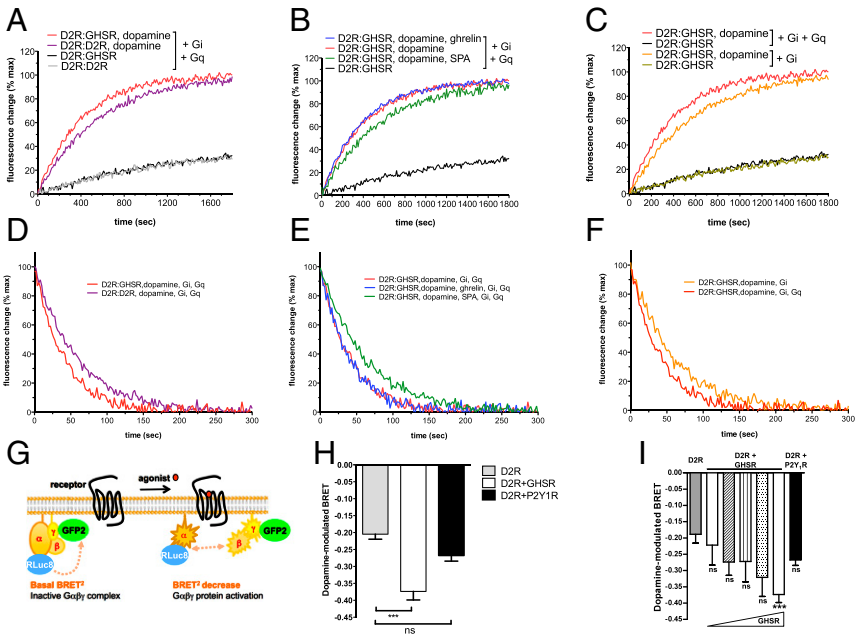

Fig. 3. Heteromer modulation of G protein activation. (*A*) GTPγS binding to $G\alpha_{i1}$ catalyzed by the D2R homomer or the GHSR:D2R heteromer in the absence or the presence of dopamine and in the presence of Gq. (*B*) GTPγS binding to $G\alpha_{i1}$ catalyzed by the GHSR:D2R heteromer in the presence of Gq and in the absence of dopamine, in the presence of dopamine only or in the presence of dopamine and 10 μM ghrelin or SPA, or (*C*) in the absence of dopamine and in the absence or presence of Gq. (*D*) FRET-monitored release of the βγ subunits of Gi from homomer- or heteromer-containing SMALPs in the presence of dopamine and Gq. (*E*) Release of Gβγ from heteromer-containing SMALPs in the presence of Gq and of dopamine or of dopamine and GHSR ligands, or (*F*) in the presence of dopamine and in the presence or absence of Gq. (*G*) Schematic representation of the BRET-based assay for $G\alpha_{i1}$ activation. (*H*) BRET signal in HEK293T/17 cells coexpressing RLuc8-$G\alpha_{i1}$, GFP2-$G\gamma_2$, and $G\beta_1$ in the presence of D2R alone or together with GHSR or $P2Y_1R$ after stimulation for 1 min with 10 μM dopamine. (*I*) BRET signal between RLuc8-$G\alpha_{i1}$ and GFP2-$G\gamma_2$ in the presence of D2R alone or together with increasing amounts of GHSR or with $P2Y_1R$ after stimulation for 1 min with dopamine (10 μM). In *H* and *I*, results are expressed as the difference in the BRET signal measured in the presence and the absence of dopamine (basal BRET signal measured in the presence of D2R alone taken as reference) and data represent the mean ± SEM of at least three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test (***$P < 0.001$; ns, not statistically significant).

was observed for the 90–238 and 171–276 pairs (Fig. 4 *B* and *C*). No significant difference was observed for the 141–333 pair (Fig. 4*D*), possibly because the distance between the probes does not fall within a region of high sensitivity in LRET. Again, the LRET profiles of the heteromer were unaffected by ghrelin, whereas the difference was abolished by SPA (*SI Appendix*, Fig. S12) or in the absence of Gq (Fig. 4).

Possible G Protein:G Protein Contacts in the Complex with the Heteromer. The effect of heteromerization on Gi was not observed in the absence of Gq, suggesting that it could be related to Gq:Gi contacts. We used molecular dynamics to tentatively assess whether such contacts could possibly occur. Because of the lack of structural information on higher-order GPCR oligomers, we restricted the model to the putative central GHSR:D2R protomers. The model was built by homology modeling using the β_2-adrenergic receptor:Gs crystal structure for the GPCR:G protein complex (28) (Fig. 5 and *SI Appendix*, Figs. S13–S15). In this orientation, the main interface between the G proteins essentially involved the two α-subunit ras-like domains (*SI Appendix*, Figs. S13*E* and S14), with the α-helical one still mobile (*SI Appendix*, Fig. S15), in agreement with LRET. Although alternative organizations can be considered (29), this model nevertheless indicates possible allosteric contacts between the G proteins. Of importance, the second GHSR or D2R protomer added to account for a tetrameric arrangement couldn't accommodate a G protein (*SI Appendix*, Fig. S16), in agreement with the experimental stoichiometry.

We then carried out a preliminary series of measurements where LRET was used as an intermolecular ruler to assess whether some of the features of the model could be compatible with the arrangement of the purified assembly. When LRET was measured between $G\alpha_q$ labeled at its N terminus with fluorescein and $G\alpha_i$ labeled with Lumi4-Tb on a unique reactive cysteine at position 289 (Fig. 5*B*), the acceptor-sensitized emission decay profile displayed a major component corresponding to a donor–acceptor distance in the 20-Å range (Fig. 5*C*). This distance was compatible with that measured from the model (in the 23-Å range) (Fig. 5 *B* and *E*). When LRET was measured between Fluo bound at position 156 in the i2 loop of GHSR (17) and Lumi4-Tb bound to position 314 of $G\alpha_i$ Hexa I, the decay curve displayed two lifetime components corresponding to donor–acceptor distances in the 35- and 60-Å ranges. Only the shorter distance was compatible with the model (38 Å) (Fig. 5 *B* and *E*). The longer one could result from LRET between $G\alpha_i$ and the second GHSR protomer, as both protomers were labeled within the heterotetramer. However, an exhaustive search procedure in the molecular dynamics experiments did not allow identifying an unambiguous interaction interface that would be compatible with the 60-Å distance (*SI Appendix*, Fig. S16), suggesting a higher dynamics of this protomer (*SI Appendix*). At the present stage, the model is thus considered a tentative one, the experimental validation of which will have to be strengthened further.

Discussion

It had been shown that GHSR and D2R form a complex in living cells that modulates dopamine-mediated signaling (9). Our results confirm the specific interaction between these two receptors even in a simplified lipid system, suggesting this is an intrinsic feature of the receptors. As for the β_2-adrenergic receptor or rhodopsin (30), oligomerization occurred only upon reconstitution in liposomes, indicating that the interaction is dependent upon lipids. Additionally, our data show that the major heteromeric species formed in liposomes is likely a tetramer composed of two each of GHSR and D2R. However, the cellular environment could further modulate this stoichiometry, as it could also affect the relative

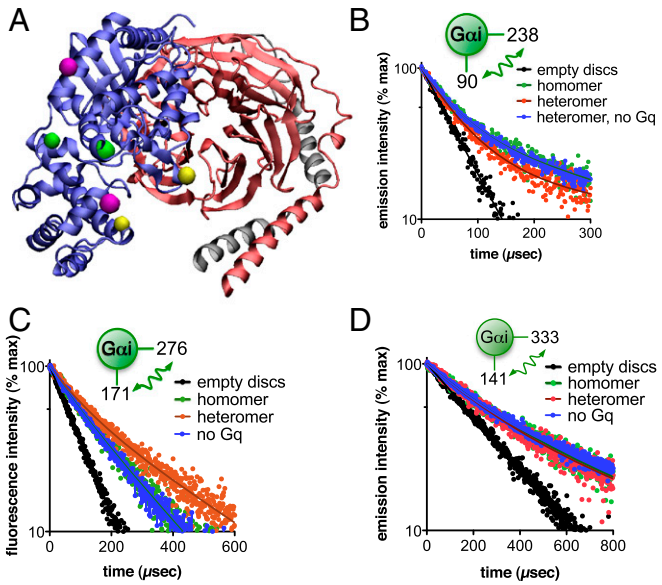

Fig. 4. Heteromer modulation of G protein conformation. (*A*) Ribbon model of Gαiβγ (1GP2) showing the position of the labeled cysteines (yellow: C^{90}, C^{238}; magenta: C^{141}, C^{333}; green: C^{276}, C^{171}). (*B–D*) Sensitized-emission decays from $G\alpha_{i1}$ labeled with Tb and fluorescein in the presence of empty SMALPs or of dopamine-activated D2R homomer or GHSR:D2R heteromer, in the absence or in the presence of Gq. The labeled positions are schematically presented in each case.

stability of the complex. Indeed, our data on receptor protomer exchange dynamics in the D2R:GHSR heteromer differ from what was reported in cellular systems for the D2R homomer. Indeed, the protomers appear to be in slow exchange in liposomes, whereas D2R was shown to form tetramers (31) with very dynamic (32) or even totally transient interactions (33) in cells. The plasma membrane is a complex environment with multiple proteins, mixtures of different lipids and cholesterol, asymmetry between the outer and inner layers, distinct domains, and no chemical equilibrium (34). This complexity can hardly be reproduced in vitro. Because both the lipid bilayer and additional proteins have been shown to modulate the association dynamics of membrane proteins through specific and nonspecific effects (35, 36), it is tempting to speculate that the differences in oligomerization dynamics result from a further modulation of the process we observed in simple lipid models by the different components of the cellular environment. These would include membrane composition and physical properties (lateral pressure, curvature elastic stress, thickness) (37), microdomains (38), interfacial lipids (39), as well as ancillary proteins (35, 40).

Association of GHSR and D2R within the same complex has been shown to affect dopamine-mediated signaling in a cellular system (9). Our data suggest that this effect on signaling could be related to an impact of GHSR:D2R association on the active conformation of $G\alpha_i$. In particular, the larger distance between LRET probes could translate a more open structure with an increased displacement of the helical vs. the nucleotide domains (27) when $G\alpha_i$ is bound to the heteromer. Alternatively, the differences in LRET efficiency could result from distinct conformational dynamics between open and closed conformations of $G\alpha_i$. This increased amplitude and dynamics could be responsible for the faster nucleotide binding rates in the in vitro model system and the related increase in dopamine-mediated Gi activation we observed in HEK cells.

These differences in $G\alpha_i$ conformation could be the consequence of specific protein:protein contacts within the $(D2R)_2$:$(GHSR)_2$:Gi:Gq assembly that would allosterically modulate the conformational landscapes of both the receptors and the G proteins. A possibility would be that apo-GHSR in the heteromer exerts an allosteric effect on the adjacent receptors that modifies D2R active conformation and its ability to trigger G protein activation. A second, nonexclusive mechanism would be to consider an influence of Gq on Gi activation, as in the GPCR priming model (41). Accordingly, the effects we observed were abolished in the absence of Gq or in the presence of the GHSR inverse agonist substance P analog that was shown to dissociate the preassembled GSHR:Gq complex. To be noted, an inverse agonist derived from substance P also abolished GHSR-dependent modulation of D2R signaling in living cells (9). However, caution needs to be exerted as, in contrast to our observations, Gq-directed siRNAs did not significantly reduce the effect of apo-GHSR on D2R signaling in cellular systems. A possibility would be that, in a cell, other G proteins that couple GHSR (G_{11}, G_{13}) (18) could take over Gq. Additional functional experiments in cellular systems and in vivo will thus be required to determine the broader physiological significance of our findings. At the present stage, our data nevertheless provide some evidence for a process where heteromerization could modulate G protein activation by promoting distinct changes in its conformation and in its conformational dynamics. This is reminiscent of what has been proposed to explain differences in G protein-activation efficacy by biased ligands of the calcitonin receptor (42), and suggests that receptor-dependent allosteric modulation of G protein conformation could be a way to extend the repertoire of mechanisms for modulating GPCR-mediated responses.

Materials and Methods

All procedures are detailed in *SI Appendix*.

Protein Preparation. GHSR, BLT1, BLT2. and 5-HT4(a) were produced in *Escherichia coli* (5, 43). D1R, D2R, and MT1R were expressed in *Pichia pastoris* (44). The soluble unlipidated mutants of $G\alpha_q$ and Gβγ were produced in *sf9*

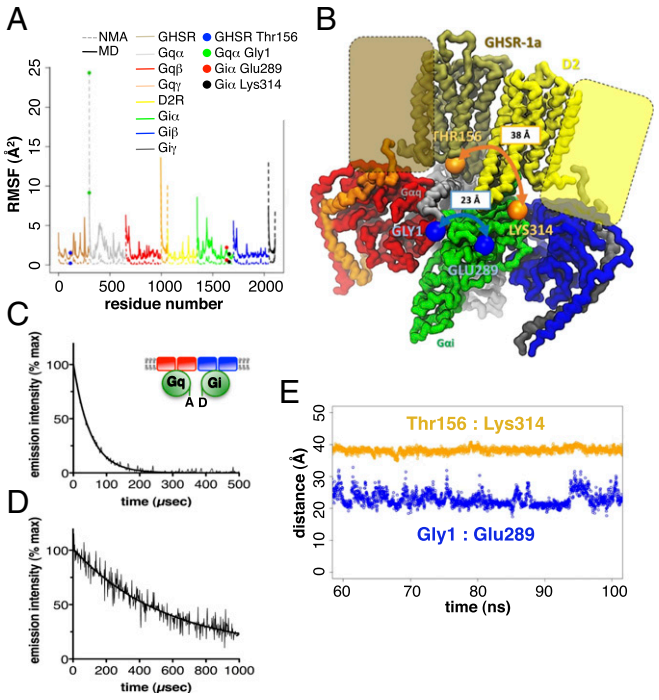

Fig. 5. The receptor:G protein model. (*A*) Root mean-squared fluctuations for each residue of the GHSR:D2R:Gq:Gi complex. Solid line: fluctuations from a 100 ns MD simulation; dotted lines: fluctuations obtained by normal mode analysis. (*B*) Model of the GHSR:D2R:Gi:Gq complex showing the positions of the LRET probes. (*C* and *D*) Sensitized-emission decays from (*C*) Tb-labeled Gi and Fluo-labeled Gq or (*D*) Tb-labeled Gi and Fluo-labeled GHSR. The different species are schematically presented (red: GHSR; blue: D2R; green: G proteins). (*E*) Time evolution of the corresponding distances along the last 50 ns of the molecular dynamics simulation.

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(22). $G\alpha_{i1}$ was expressed in *E. coli* (27) and 5HW incorporated during bacterial expression (20).

Protein Labeling. $GHSRC^{304}$ or $BLT1C^{266}$ were labeled with fluorescein-(ThermoFisher), Cy3-, or Cy5-maleimide (GE Healthcare) by incubating them with the dye at 4 °C for 16 h. For LRET, GHSR was labeled with Click-IT Alexa Fluor 488 DIBO Alkyne (Life Technologies) (18) and $G\alpha_i$ Hexa I by adding the fluorophores in a stepwise manner. $G\alpha_q$, $G\alpha_{i1}$, and $G\beta\gamma$ were labeled on their N terminus using the NHS derivative of the fluorophore (17, 21).

Proteoliposome Assembly. Receptors were inserted into β-DDM solubilized 200 nm DOPC, DOPE, DOPS (40/40/20) (Avanti lipids) liposomes containing cholesterol (0.2 cholesterol-to-lipid molar ratio) (13) and further purified on a 5–30% linear sucrose gradient.

FRET, HTRF, LRET, and BRET Measurements. Fluorescence emission spectra were recorded on a Cary Eclipse spectrofluorimeter (Varian) with an excitation at 495 nm. For HTRF-monitored dimerization assays, the signal was optimized by varying concentrations in the acceptor-labeled antibody at fixed donor concentration (45). In LRET assays, the donor lifetimes in the presence of the acceptor were measured through the acceptor-sensitized emission at 515 nm (λ_{exc}: 337 nm). G protein-activation experiments in HEK293T/17 cells were performed as previously described (23–25).

Liposome Solubilization and SMALP Preparation. The proteoliposomes in 50 mM Tris·HCl, 200 mM NaCl pH 8 were incubated at 25 °C with the SMA copolymer at a lipid-to-polymer molar ratio of 0.10. The S-tag of GHSR and the Flag-tag of D2R were used to purify heteromer-containing SMALPs (20).

Chemical Cross-Linking. Proteins in SMALPs were cross-linked in the presence of DMS to a final concentration of 1 mg/mL. The reaction was stopped by addition of glycine to a concentration of 50 mM and the cross-linked species submitted to SDS/PAGE after extraction by $CH_3OH/CHCl_3/H_2O$ (15).

GTPγS Binding and G Protein Dissociation Assays. GTPγS binding experiments were carried out using Bodipy-FL GTPγS (5). The rate of GTPγS binding to Gi was determined by monitoring the relative increase in the intrinsic 5HW fluorescence (λ_{exc}: 315 nm; λ_{em}: 350 nm) as a function of time after the addition of GTPγS.

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