

Functional Selective Oxytocin-derived Agonists Discriminate between Individual G Protein Family Subtypes^{*[5]}

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Background: The oxytocin receptor couples to multiple G proteins, leading to different physiological responses.

Results: We screened for functional selective oxytocin receptor agonists and identified two analogs that activate individual G_i subunits.

Conclusion: Functional selective analogs discriminate among different receptor conformations coupled to G_i proteins.

Significance: These compounds will contribute to the development of selective drugs with new selectivity and therapeutic profiles.

We used a bioluminescence resonance energy transfer biosensor to screen for functional selective ligands of the human oxytocin (OT) receptor. We demonstrated that OT promoted the direct engagement and activation of G_q and all the G_{i/o} subtypes at the OT receptor. Other peptidic analogues, chosen because of specific substitutions in key OT structural/functional residues, all showed biased activation of G protein subtypes. No ligand, except OT, activated G_{oA} or G_{oB}, and, with only one exception, all of the peptides that activated G_q also activated G_{i2} and G_{i3} but not G_{i1}, G_{oA}, or G_{oB}, indicating a strong bias toward these subunits. Two peptides (DNalOVT and atosiban) activated only G_{i1} or G_{i3}, failed to recruit β -arrestins, and did not induce receptor internalization, providing the first clear examples of ligands differentiating individual G_{i/o} family members. Both analogs inhibited cell proliferation, showing that a single G_i subtype-mediated pathway is sufficient to prompt this physiological response. These analogs represent unique tools for examining the contribution of G_{i/o} members in complex biological responses and open the way to the development of drugs with peculiar selectivity profiles. This is of particular relevance because OT has been shown to improve symptoms in neurodevelopmental and psychiatric disorders characterized by abnormal social behaviors, such as autism. Functional selective ligands, activating a specific G protein signaling pathway, may possess a higher efficacy and specificity on OT-based therapeutics.

It was long believed that G protein-coupled receptors (GPCRs)³ work as bimodal switches between an agonist-promoted “on” state, capable of engaging a specific G protein isoform, and an uncoupled “off” state. However, more recent evidence indicates that GPCR signaling is much more complex than originally thought; a single receptor subtype can activate multiple G protein-dependent and/or G protein-independent effectors, and different agonists can activate the different effectors with different intrinsic efficacies. The most widely accepted theory explaining the signaling complexity of GPCRs is that they adopt a range of distinct conformations that are differentially stabilized or induced by various endogenous or synthetic agonists; this ligand-induced activation of independent signaling conformations has been called “functional selectivity” (1). It has been shown that many ligands acting at GPCRs are characterized by functional selectivity, and the number of

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³ The abbreviations used are: GPCR, G protein-coupled receptor; AVP, arginine vasopressin; BRET, bioluminescence resonance energy transfer (BRET¹ uses RLuc as a donor, the h derivative of coelenterazine as its substrate, and YFP as the acceptor; BRET² uses RLuc as a donor, the coelenterazine 400a coelenterazine derivative as its substrate, and GFP¹⁰ as the acceptor); G α_x , a G protein α subunit subtype (G α_q , G α_{11} , etc.); G α_q -97-RLuc, α_q subunit in which RLuc was inserted in position 97; G α_{11} -91-RLuc, α_{11} subunit in which RLuc was inserted in position 91; G α_{12} -91-RLuc, α_{12} subunit in which RLuc was inserted in position 91; G α_{13} -91-RLuc, α_{13} subunit in which RLuc was inserted in position 91; G α_{oA} -91-RLuc8, G α_{oA} subunit in which RLuc8 was inserted in position 91; G α_{oB} -91-RLuc8, G α_{oB} subunit in which RLuc8 was inserted in position 91; G α_s -113-RLuc8, G α_s subunit in which RLuc8 was inserted in position 113; G β_x , a G protein β subunit subtype (G β_1 , etc.); G γ_x , a G protein γ subunit subtype (G γ_1 , etc.); G γ_x , a heterotrimeric complex containing α_x as the α subunit (e.g. G α_q is a heterotrimeric complex containing α_q as the α subunit); GFP¹⁰, a blue-shifted variant of A. victoria GFP; HEK293, human embryonic kidney 293 cell line; HTRF, homogeneous time-resolved FRET; IP1, myo-inositol-1-phosphate; OT, oxytocin; OTR, oxytocin receptor; hOTR, human OTR; PTX, pertussis toxin; RLuc, Renilla luciferase; RLuc8, a Renilla luciferase mutant with a total of eight favorable mutations; V1a, vasopressin receptor subtype 1a; V1b, vasopressin receptor subtype 1b; V2, vasopressin receptor subtype 2; TM, transmembrane helix; ANOVA, analysis of variance; AVT, arginine vasotocin.

“functional selective ligands” has rapidly increased over recent years; however, the structural characteristics underlying functional selectivity are still little understood. In particular, it will be important to determine, at individual GPCRs, the molecular basis of the coupling efficiency of individual ligands to different G protein subtypes and effectors in order to define their potential use in specific cell contexts.

The oxytocin receptor (OTR) is a GPCR whose promiscuous coupling to G_q and G_i heterotrimeric complexes has been described in several cell types (2–5). In different cell systems, the multiple signaling pathways activated by OTRs may act synergistically (as in the case of the contraction induced in myometrial cells by OTR coupling to $G_{\alpha_{q-11}}$ and to the small G proteins of the Rho family) (6). However, they may also have opposite effects on the same cell function, as in the case of neuronal cells in which OT can inhibit (via a PTX-resistant G protein pathway) or stimulate (via a PTX-sensitive G protein pathway) K^+ conductances belonging to the inward rectifier family of K^+ channels (4). Similarly, in human embryonic kidney HEK293 cells stably transfected with human OTRs, receptor coupling to G_i is responsible for inhibiting cell growth, whereas receptor coupling to a pertussis toxin (PTX)-insensitive complex (possibly G_q) stimulates cell growth (5, 7).

Because of this heterogeneity in the final outcome of receptor activation, functional selective ligands will be of great help in identifying the roles of the different OTR-elicited pathways in physiological functions; moreover, as they may have distinct therapeutic actions, they may lead to new therapeutic approaches. In the case of OTR-expressing tumors, the activation of specific OTR- G_i signaling inhibits cell growth (8) and stops cell migration (9), and in line with these findings, it has been shown that atosiban, identified in our laboratory as a biased agonist that favors $G_{i/o}$ over G_q coupling (8), inhibits the growth of human prostate adenocarcinoma cells *in vitro* (8) and rat and mouse mammary carcinomas *in vitro* and *in vivo* (10). The use of OTR- G_i functional selective ligands therefore seems to be a promising means of inducing cancer regression and preventing breast and prostate cancer invasion and metastases. Furthermore, it has recently been suggested that the intranasal administration of OT can be used to promote prosocial behavior and decrease anxiety in patients with neurodevelopmental and psychiatric disorders, such as autism and schizophrenia (11, 12). However, the signaling pathways underlying the physiological effects induced by OT in neuronal cells are still largely unknown. The possibility of pharmacologically manipulating OT-induced neurophysiological functions by activating defined signaling cascades should help in the development of innovative OT-based therapeutic protocols.

To develop functional selective OTR ligands and fully exploit their potential, a number of questions concerning the functional coupling of OTRs need to be answered. Which G protein complexes can OTRs couple to? What is the efficiency of OTR coupling to the different G protein complexes? Which pathways can functional selective ligands be effective on? What are the structural features characterizing these analogues? To start answering these questions, we used a bioluminescent reso-

nance energy transfer (BRET)-based biosensor⁴ to screen a number of OT/AVP-derived peptides for their ability to activate G_q , G_{i1} , G_{i2} , G_{i3} , G_{oA} , G_{oB} , and G_s -transducing complexes.

We found that all of the tested AVP and OT analogues harboring substitution in functionally important domains of the peptides activate G_q , G_{i2} , and G_{i3} with comparable relative efficacy, but none of them (not even those that activate the other G_i members as effectively as OT) can reliably activate G_{i1} , G_{oA} , and G_{oB} , thus indicating a bias toward these subunits; furthermore, two compounds (DNalOVT and atosiban) were entirely biased toward G_{i1} or G_{i3} activation, representing the first examples of biased ligands differentiating $G_{i/o}$ subtypes. We also found that the G_i functional selective ligands generated G protein activation without β -arrestin recruitment or OTR internalization, thus indicating that they also have a bias toward β -arrestin activity.

EXPERIMENTAL PROCEDURES

Reagents, Constructs, and Peptides—The coelenterazine h came from Molecular Probes, Invitrogen (Milan, Italy), coelenterazine 400a (CLz400) from Biotium (Hayward, CA), and PTX from Sigma-Aldrich. The expression vector for G proteins fused to *Renilla* luciferase G_{α_q-97} -Rluc, $G_{\alpha_{i1}-91}$ -Rluc, $G_{\alpha_{i2}-91}$ -Rluc, $G_{\alpha_{i3}-91}$ -Rluc, $G_{\alpha_{oA}-91}$ -Rluc8, $G_{\alpha_{oB}-91}$ -Rluc8, and G_{α_s-113} -Rluc8 cDNAs are described elsewhere.⁴ The G_{α_q} , $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, G_{α_s} , $G_{\alpha_{oA}}$, $G_{\alpha_{oB}}$, and G_{γ_2} cDNAs came from the Missouri S&T cDNA Research Center (Rolla, MO). The plasmids encoding GFP¹⁰- G_{γ_2} and $G\beta_1$ have been described previously (13); the plasmids encoding for the human OTR and V₂R are described in Refs. 14 and 15; and OTR-Rluc, OTR-YFP, and OTR-EGFP are as in Refs. 7 and 16. OTR-GFP² was obtained by subcloning the cDNA sequence of the OTR into the pGFP²-N2 vector (PerkinElmer Life Sciences). Briefly, the OTR in pEGFP-N3 was amplified by PCR using forward (5'-CAAAAAGCTTATGGAGGGCGCGCTCGCAG-3') and reverse (5'-GTTTGGATCCCGTGGATGGCTGGGAGCAG-3') primers, and the resulting PCR product was subcloned into the pGFP²-N2 vector; the construct was confirmed by bidirectional sequencing. The CD4-GFP¹⁰ vector is described elsewhere (17). The expression vector for β -arrestin2-YFP (originally developed in the laboratory of M. Bouvier) came from Dr. J. Perroy (Institut de Génomique Fonctionnelle, Montpellier, France), and the expression vector for β -arrestin1-YFP was from Dr. C. Hoffmann (University of Würzburg). β -Arrestin2-Rluc and Rluc- β -arrestin2 have been described previously (18, 19). OT, AVP, AVT, and atosiban came from Bachem (Weil am Rhein, Germany). All of the other peptides used in this study were synthesized as in Refs. 20 and 21.

Cell Cultures and Transfection—The DU145 human prostate carcinoma, HEK293, and COS7 cell lines were purchased from the American Type Culture Collection (Manassas, VA). HEK293 cells stably expressing the human OTR cDNA C-terminally fused to EGFP or N-terminally tagged to *c-myc* have been described elsewhere (5, 7, 22). For transfection, cells were

⁴ A. Sauliere, M. Bellot, H. Paris, C. Denis, F. Finana, M. F. Altié, M. H. Seguelas, J. L. Hansen, J. M. Sénard, and C. Galès, submitted for publication.

seeded at a density of 3,100,000 cells/well in 100-mm plates on the day before transfection. A mix containing 20 μg of DNA and 60 μg of polyethylenimine (PEI linear, M_r 25,000, Polysciences Europe GmbH, Eppelheim, Germany) was prepared with 1 ml of basic medium (without additives such as serum or antibiotics) and, after 15 min of incubation at room temperature, added directly to cells maintained in 10 ml of complete medium containing 10% FBS. 24 h after transfection, the supplemented DMEM was renewed, and the cells were cultured for a further 24 h before the experiments. 48 h after transfection, the cells were washed twice, detached, and resuspended with PBS, 0.5 mM MgCl_2 at room temperature.

Ligand Binding Assays—The binding assays were performed at 30 °C on membranes prepared from COS7 cells transiently transfected by means of electroporation with the wild-type human OTR (23, 24), using the radiolabeled OTR receptor agonist [^3H]OT (PerkinElmer Life Sciences); peptide affinities (K_i) were determined by means of competition experiments in which the peptide concentrations varied from 10^{-11} to 10^{-6} M, and the concentration of the radioligand was 4×10^{-9} M. Nonspecific binding was determined in the presence of unlabeled OT (10^{-3} M). The ligand binding data (K_i) were analyzed by means of non-linear regression, one-site binding competition fit using GraphPad Prism software, version 5 (GraphPad, Inc., San Diego, CA).

BRET Assay—To detect and analyze the interactions between OTR and the different $G\alpha$ subunits by means of BRET² experiments, HEK293 cells were co-transfected with OTR-Rluc, GFP¹⁰- $G\gamma_2$, $G\beta_1$, and one of $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{oA}$, or $G\alpha_{oB}$. To screen for the effects of the different ligands on G protein activation, HEK293 cells were co-transfected with $G\alpha_q$ -97-Rluc, $G\alpha_{i1}$ -91-Rluc, $G\alpha_{i2}$ -91-Rluc, $G\alpha_{i3}$ -91-Rluc, $G\alpha_{oA}$ -91-Rluc8, $G\alpha_{oB}$ -91-Rluc8, and $G\alpha_s$ -113-Rluc8 constructs in the presence of plasmids encoding for GFP¹⁰- $G\gamma_2$, $G\beta_1$, and the OTR or V₂R. Finally, to study OTR-mediated β -arrestin recruitment by means of BRET¹ experiments, the cells were co-transfected with OTR-Rluc and β -arrestin1-YFP or β -arrestin2-YFP or with OTR-YFP and β -arrestin2-Rluc or Rluc- β -arrestin2. 48 h after transfection, the cells were washed twice, detached, and resuspended with PBS, 0.5 mM MgCl_2 at room temperature. They were then distributed in a white 96-well microplate (100 μg of proteins/well) (Optiplate, PerkinElmer Life Sciences) and incubated in the presence or absence of different concentrations of OT or different ligands for 2 min before substrate addition.

The BRET between Rluc/Rluc8 and GFP¹⁰ was measured immediately after the addition of the Rluc substrate coelenterazine 400a (5 μM), using an Infinite F500 reader plate (Tecan, Milan, Italy) that allows the sequential integration of light signals detected with two filter settings (Rluc/Rluc8 filter, 370–450 nm; GFP¹⁰ filter, 510–540 nm). The data were recorded, and the BRET² signal was calculated as the ratio between GFP¹⁰ emission and the light emitted by Rluc/Rluc8. The changes in BRET induced by the ligands were expressed on graphs as “ligand-promoted BRET” using the formula,

$$\text{Ligand-promoted BRET} = \left(\frac{\text{emission GFP}^{10}_{\text{ligand}}}{\text{emission Rluc}_{\text{ligand}}} - \left(\frac{\text{emission GFP}^{10}_{\text{PBS}}}{\text{emission Rluc}_{\text{PBS}}} \right) \right) \quad (\text{Eq. 1})$$

For titration experiments, HEK293 cells were transfected using a constant amount of $G\alpha_s$ -113-Rluc8 with increasing amounts of OTR-GFP² or CD4-GFP¹⁰ vectors.

The expression level of each tagged protein was determined by direct measurement of total fluorescence and luminescence in an aliquot of the transfected cells using an Infinite F500 reader plate (Tecan). Total GFP² or GFP¹⁰ fluorescence was measured using an excitation filter at 400 nm and an emission filter at 510 nm. After fluorescence measurement, the same cell sample was incubated for 8 min with 5 μM coelenterazine h, and the total luminescence was measured.

To analyze the kinetics of the OTR- β -arrestin interactions, coelenterazine h (the substrate specific for BRET¹ experiments) was added 8 min before the addition of the different ligands, and readings were made every 20 s using an Infinite F500 reader plate (Tecan) and filter set (Rluc filter, 370–480 nm; YFP filter, 520–570 nm). To determine the half-time ($t_{1/2}$) of OT- and other ligand-induced BRET, the data were recorded as the difference between the ligand-promoted BRET signal and the average of the base-line (PBS-treated) BRET signal, and the time at which the half-BRET peak was reached was estimated. To produce the dose-response curves of OT-induced β -arrestin recruitment, the cells were preincubated with coelenterazine h and treated with increasing concentrations of OT; the BRET signal for each peptide dose was recorded at the maximum BRET peak, which corresponds to 5 min for β -arrestin1 and 2 min for β -arrestin2.

Inositol Phosphate Measurements—IP1 accumulation in HEK293 cells stably transfected with OTR (100,000 cells) was determined in 96-well half-area microplates (Corning Glass) using the HTRF-IP-One kit (CisBio International, Bagnols-sur-Cèze, France). The time-resolved FRET signals were measured 50 s after excitation at 620 and 665 nm using a Tecan Infinite F500 instrument. The IP1 concentrations were interpolated from the IP1 standard curve supplied with the kit.

Cell Growth Assay—Experiments were carried out in the log phase of growth after the cells had been seeded in 96-well plates (3,000 cells/well) and allowed to adhere for 24–48 h. OT, DNalOVT, and atosiban were added to the medium for 48 or 72 h at a final concentration of 100 nM, and cell growth was determined using an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS)-based assay (CellTiter 96[®] Aqueous One Solution Assay, Promega, Milan, Italy). Where indicated, the cells were exposed to PTX for 16 h before treatment with DNalOVT and atosiban. All of the treatments were performed in sextuplicate, and a linear correlation between absorbance and cell counts was established for up to 20,000 cells. Cell growth variation was expressed as the percentage difference between the treated and untreated cells (set at 100%).

Fluorescent Microscopy—To detect OTR internalization, HEK293 cells stably transfected with OTR-EGFP were stimulated with OT (100 nM), atosiban (100 nM), and DNalOVT (100 nM) for 3 or 30 min under controlled conditions (37 °C, 10% CO_2). The stimulation was blocked by putting the dishes containing the cells on ice and washing the cells three times with ice-cold PBS/ Ca^{2+} / Mg^{2+} (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.1 mM CaCl_2 , 1 mM MgCl_2 , pH

7.4). The cells were fixed with 4% paraformaldehyde for 20 min at room temperature and washed twice with PBS/Ca²⁺/Mg²⁺ and once with H₂O, and the glass coverslips were mounted with MOWIOL. The cells were analyzed using an LSM 510 META confocal laser-scanning microscope (Zeiss, Jena, Germany) and the following filter set: XF1042, 485DF15 (excitation); XF2043, 490–550DBDR (dichroic); and XF3056, 530–580DBEM (emission).

Statistical Analysis—All of the data were analyzed using GraphPad Prism software, version 5 (GraphPad, Inc.) and are given as the mean values $\pm$ S.D. of at least three independent experiments. One-way ANOVA followed by Dunnett's *post hoc* test was used to determine statistically significant differences in the ligand-induced BRET ratio *versus* PBS-stimulated cells, IP1 accumulation in treated *versus* untreated cells, and variations in cell proliferation of ligand-stimulated cells *versus* untreated cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Concentration-response experiments were analyzed to non-linear curve fitting using the sigmoidal dose-response equation. The kinetics data were normalized by setting the zero time point immediately after the addition of the ligand; the data were analyzed by means of non-linear least-square fitting to the one-phase exponential association equation.

RESULTS

OT Interacts and Activates $G\alpha_q$, $G\alpha_p$, and $G\alpha_o$ Subunits—To investigate the coupling specificity of human OTRs, we first performed BRET² experiments to measure the OT-induced BRET signal between the OTRs and a number of G protein isoforms. As shown in Fig. 1a, these experiments were performed using HEK293 cells that were transiently transfected with the OTR C-terminally fused with the BRET energy donor *Renilla reniformis* luciferase (OTR-RLuc), the $G\gamma_2$ subunit N-terminally fused with a blue-shifted variant of *Aequorea victoria* green fluorescent protein (GFP¹⁰- $G\gamma_2$), $G\beta_1$, and one of the seven different $G\alpha$ subunits ($G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, or $G\alpha_s$) (25). The cells were then stimulated for 2 min with 10 μ M OT, and the BRET signal was monitored. As shown in Fig. 1a, a statistically significant ($p < 0.001$) OT-induced increase in the BRET signal was observed in the presence of $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, and $G\alpha_{oB}$, thus indicating the interaction of the OTR with the G_q , G_i , and G_o complexes; no statistically significant increase in the BRET signal was observed after co-expression of the $G\alpha_s$ subunit.

Because previous work in the literature reported OTR- $G\alpha_s$ association (3), we checked if any specific preformed interaction resulting in stable proximity between the donor and acceptor could have masked an agonist-induced BRET increase. To this aim, we performed a BRET titration assay in which we progressively increased the amount of OTR-GFP² or of a negative control CD4-GFP¹⁰ over a fixed amount of $G\alpha_s$ -113-Rluc8 (Fig. 1b). No differences between the BRET ratio of OTR-GFP² and that of the negative control CD4-GFP¹⁰, which is plasma membrane-located as the OTR but does not specifically interact with G protein complexes, were found. Moreover, the fact that the BRET ratio values obtained with OTR-GFP² and CD4-GFP¹⁰ were fitted by a first order curve indicated similar, non-

specific, protein-protein interactions of OTR-GFP² and CD4-GFP¹⁰ with $G\alpha_s$ -113-Rluc8 (26).

In conclusion, although the biosensor used above reports agonist-induced receptor/G protein interactions without giving insight into the underlying process of G protein activation, it was nevertheless a potent indicator of G protein coupling selectivity (25), and its use allowed us to define the specific G proteins physically engaged by OTRs even in overexpression conditions.

In order to demonstrate the ligand-induced activation of the different G protein complexes more directly, we then used a BRET biosensor in which the energy transferred between the $G\alpha$ and $G\gamma$ subunits of the heterotrimeric G-protein complex accurately measures the separation of the $G\alpha$ and $G\beta\gamma$ subunits that follows receptor activation (13). The energy donor (Rluc) is inserted within the $G\alpha$ subunit amino acid sequence, and the acceptor (GFP¹⁰) is N-terminally fused to the $G\gamma_2$ subunit (GFP¹⁰- $G\gamma_2$). Previously engineered to measure $G\alpha_{i1}$ activation (13), BRET probes have now been built for all of the $G\alpha$ protein isoforms.⁴

An $G\alpha_s$ -113-Rluc8 was first used to confirm that OT is unable to induce OTR- G_s activation. No change in BRET was detected with increasing OT concentrations up to 10^{-5} M, whereas a significant ($p < 0.001$) BRET decrease was obtained with the positive control V₂R stimulated with its natural agonist AVP (10^{-5} M) (Fig. 1c).

We then investigated OTR coupling to $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, and $G\alpha_{oB}$ with the $G\alpha_q$ -97-Rluc, $G\alpha_{i1}$ -91-Rluc, $G\alpha_{i2}$ -91-Rluc, $G\alpha_{i3}$ -91-Rluc, $G\alpha_{oA}$ -91-Rluc8, and $G\alpha_{oB}$ -91-Rluc8 constructs; Rluc8 (27) constructs were used to characterize $G\alpha_{oA}$ and $G\alpha_{oB}$ functional selectivity because the agonist-promoted BRET variation was more pronounced and less variable with them than with the Rluc.⁴ To avoid possible variations in the BRET signal resulting from fluctuation in the relative expression level of donors and acceptors, we set up transfection conditions in which comparable protein expression levels were maintained constant (see supplemental Fig. 1, a and b). Very similar values of total luminescence were indeed obtained for all $G\alpha$ -Rluc constructs (mean $24,390 \pm 841.5$ arbitrary units). In the case of $G\alpha$ -Rluc8 constructs, 4-fold higher values were obtained ($104,300 \pm 1,793$ arbitrary units); because the Rluc8 enzyme has been shown to produce a 4-fold improvement in light output (28), these data indicate that in our experimental conditions, Rluc8 expression is almost identical to that of Rluc and that the levels of expression of all $G\alpha$ subunits are comparable.

Using these probes and experimental conditions, we found that OTRs not only recruit but also activate $G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, and $G\alpha_{oB}$, as demonstrated by the decrease in the BRET signal ratio measured for all of the tested G protein isoforms following activation by OT (Fig. 1d). Furthermore, as shown in Fig. 2, this decrease in the BRET ratio was OT concentration-dependent in all different $G\alpha$ subunit-transfected cells.

Screening of Functional Selective Ligands; Identification of Functional Selective $G\alpha_{i1}$ and $G\alpha_{i3}$ Analogues—In order to find and characterize new OTR coupling-selective analogues, we screened a series of OT- and atosiban-derived peptides whose

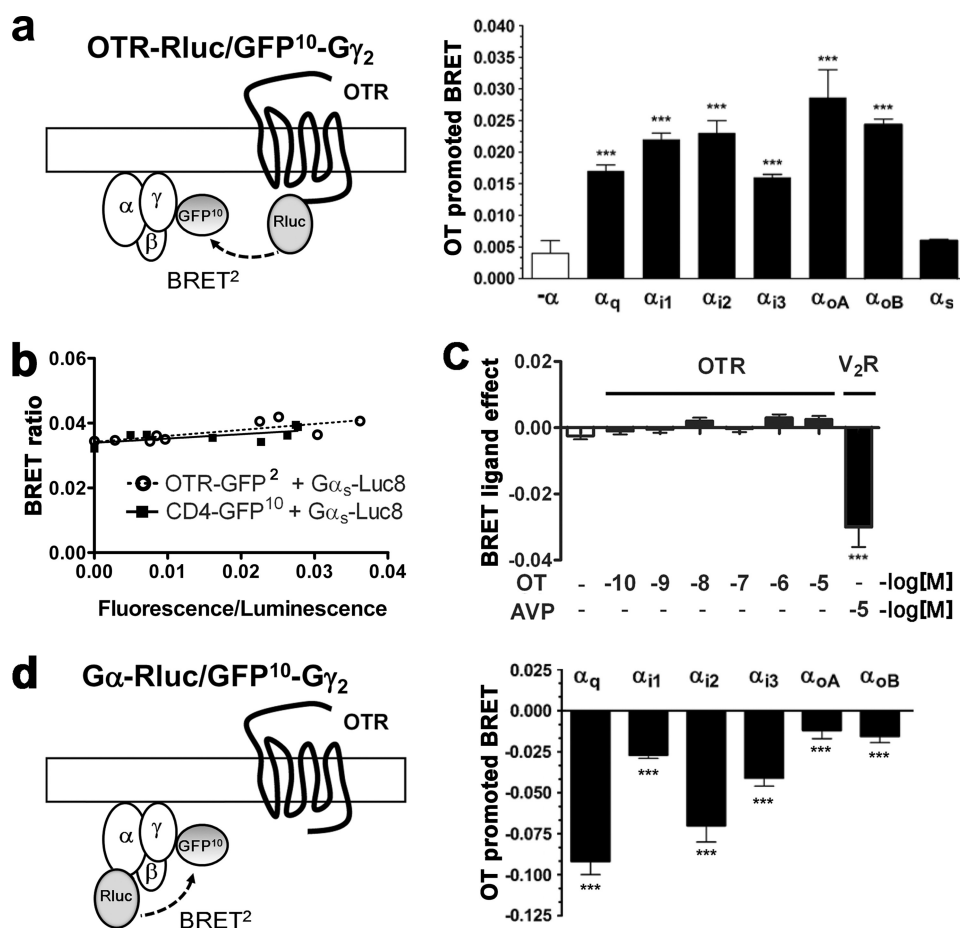


FIGURE 1. BRET measurements of OTR-Gαβ₁γ₂ coupling and activation following OT stimulation. *a*, BRET² was measured between Rluc (the donor) and GFP¹⁰ (the acceptor), respectively, introduced at the C-terminal tail of OTR (OTR-Rluc) and the N-terminal domain of the Gγ₂ subunit (GFP¹⁰-Gγ₂). OT-induced OTR-Gα coupling places OTR-Rluc and GFP¹⁰-Gγ₂ near each other, which corresponds to an increase in the GFP¹⁰/Rluc BRET ratio. BRET was measured in HEK293 cells co-expressing OTR-Rluc, GFP¹⁰-Gγ₂, and Gβ₁ in the absence (−α, empty bar) or presence of the indicated Gα subunits (*n* = 4). The results are the differences in the BRET signal with OT (10 μM) or PBS (mean value ± S.D. of three independent experiments). One-way ANOVA followed by Dunnett's test was used to determine the statistical differences between OT-promoted BRET in the presence of the indicated Gα proteins and non-Gα-transfected controls (***, *p* < 0.001). *b*, a BRET titration curve was performed in HEK 293 cells transiently transfected to co-express Gα_s-113-Rluc8 and OTR- GFP² or CD4- GFP¹⁰ in combination with β₁γ₂ subunits. The amount of plasmid encoding GFP²-tagged proteins varied (from 0.031 to 8 μg), whereas the amount of Gα_s-113-Rluc8 was kept constant (3 μg). Data are representative of two experiments and were fit using linear regression. *c*, Gα_s activation was evaluated with BRET in HEK293 cells co-expressing OTR or the positive control V₂R with GFP¹⁰-Gγ₂, Gβ₁, and Gα_s-Rluc8 tagged subunits. Cells were stimulated with OT or AVP at the concentration indicated. The results are the differences in the BRET signal with OT, AVP, or PBS (empty bar) (mean values ± S.D. (error bars) of three independent experiments). One-way ANOVA followed by Dunnett's test was used to determine the statistical differences between ligand-promoted BRET in the presence of the indicated ligand and untreated controls (***, *p* < 0.001). *d*, BRET² was measured between Rluc (the donor) and GFP¹⁰ (the acceptor), introduced into the α helical domain of the indicated Gα subunits and the N-terminal domain of Gγ₂ (GFP¹⁰-Gγ₂), respectively. Ligand-induced OTR-Gα activation leads to a conformational rearrangement of the heterotrimeric G protein complex that corresponds to a decrease in the BRET ratio. BRET was measured in HEK293 cells co-expressing OTR, GFP¹⁰-Gγ₂, Gβ₁, and Rluc/Rluc8-tagged Gα subunits: α_q (*n* = 6), α_{i1} (*n* = 14), α_{i2} (*n* = 5), α_{i3} (*n* = 8), α_{oA} (*n* = 3), and α_{oB} (*n* = 3). The results are the differences in the BRET signal with OT (10 μM) or PBS and are expressed as mean values ± S.D. One-way ANOVA followed by Dunnett's test was used to determine the statistical differences between OT-promoted BRET in the presence of the indicated Gα proteins and untreated controls (base line) (***, *p* < 0.001).

amino acid sequences and affinities for the human OTR are shown in Table 1. For the peptides whose binding affinity for human OTR was not available in the literature (Thr⁴OT, Thr⁴OVT, dThr⁴OVT, DTyrOVT, and DThiOVT), *K_i* values were determined by means of [³H]OT competition binding experiments using transiently transfected COS7 cells (supplemental Fig. 2).

The rationale underlying the choice of these analogues is based on some of their previously reported pharmacological properties. First of all, OT, AVP, Phe³OT, AVT, and dLVT were selected as a group of peptides that differ at residues 3 and 8, two positions that are known to contribute to the peptides' high affinity and potency for the different OT/AVP receptor sub-

types (14, 20). Second, to identify the residue(s) that contribute to converting the unselective G_q/G_{i/o} endogenous ligand OT into the functional selective G_{i/o} analog atosiban, we separately and singly introduced into OT all of the substitutions that finally lead to atosiban, in which the Tyr in position 2 is replaced by O-ethyl-D-tyrosine (D-Tyr(Et)) to obtain the Thr⁴OT, Thr⁴OVT, and dThr⁴OVT analogues. Third, given the putative relevance of position 2 in atosiban, we also used four known peptides that bear different substitutions at this position: Tyr(Me)OVT, DTyrOVT, DNalOVT, and DThiOVT. It has been reported that these peptides are OTR-Gα_q antagonists (20), and we speculated that they may reveal biased activity.

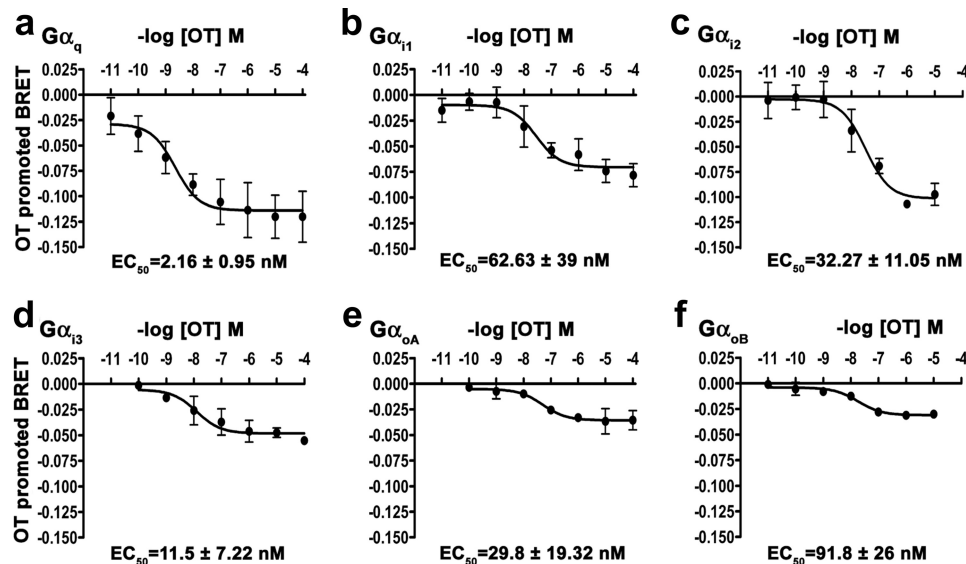


FIGURE 2. BRET measurements of the OT dose-response activation of OTR-Gαβ1γ2 complexes in HEK293 cells. BRET was measured in HEK293 cells co-expressing the indicated Gα Rluc/Rluc8 proteins (Gα_q-97-Rluc (a), Gα_{i1}-91-Rluc (b), Gα_{i2}-91-Rluc (c), Gα_{i3}-91-Rluc (d), Gα_{oB}-91-Rluc (e), and Gα_{oA}-91-Rluc (f)), together with OTR, GFP¹⁰-Gγ₂, and Gβ₁. The GFP¹⁰/Rluc ratios were similar for all pairs: 0.66 for Gα_q-97-Rluc, 0.89 for Gα_{i1}-91-Rluc, 0.65 for Gα_{i2}-91-Rluc, 0.68 for Gα_{i3}-91-Rluc, 0.59 for Gα_{oB}-91-Rluc, and 0.61 for Gα_{oA}-91-Rluc. The cells were stimulated with increasing concentrations of OT (from 10⁻¹¹ to 10⁻⁴ M) for 2 min. The results are the differences in the BRET signal in the presence and absence of OT and are expressed as the mean value ± S.D. (error bars) of at least three independent determinations. Calculated EC₅₀ values were 2.16 ± 0.95, 62.63 ± 39.00, 32.27 ± 11.05, 11.50 ± 7.22, 29.80 ± 19.32, and 91.80 ± 26.00 nM, respectively.

TABLE 1
OT- and atosiban-derived peptides

Substitutions and/or modifications of the amino acid sequence of OT are indicated in boldface type; the superscript numbers indicate the position of the residue in the peptide sequence. d, deamino; d-Tyr (Et), O-ethyl-D-tyrosine; desGly-NH₂, desglycineamide; d(CH₂)₅, β-mercapto-β,β-pentamethylenepropionic; Tyr(Me), O-methyl tyrosine; D-2-Nal, D-2-naphthylalanine; D-Thi, D-β-thienylalanine; Orn, ornithine. Reported affinities (K_i) were determined in COS7 cells, with only the exception of Tyr(Me)OVT and DNalOVT, for which CHO cell expression systems were used. *, this publication.

Peptide name	Peptide sequence	Affinity (K _i) (nM)	Reference
OT	Cys ¹ , Tyr ² , Ile ³ , Gln ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Leu ⁸ , Gly-NH ₂ ⁹	0.79	(14)
AVP	Cys ¹ , Tyr ² , Phe ³ , Gln ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Arg ⁸ , Gly-NH ₂ ⁹	1.65	(14)
AVT	Cys ¹ , Tyr ² , Ile ³ , Gln ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Arg ⁸ , Gly-NH ₂ ⁹	0.36	(14)
Phe ³ OT	Cys ¹ , Tyr ² , Phe ³ , Gln ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Leu ⁸ , Gly-NH ₂ ⁹	1.54	(14)
dLVT	dCys ¹ , Tyr ² , Phe ³ , Gln ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Lys ⁸ , Gly-NH ₂ ⁹	3.88	(50)
Thr ⁴ OT	Cys ¹ , Tyr ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Leu ⁸ , Gly-NH ₂ ⁹	0.49	*
Thr ⁴ OVT	Cys ¹ , Tyr ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Gly-NH ₂ ⁹	9.21	*
dThr ⁴ OVT	dCys ¹ , Tyr ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Gly-NH ₂ ⁹	2.51	*
atosiban	dCys ¹ , D-Tyr (Et) ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Gly-NH ₂ ⁹	52.0	(51)
Tyr(Me)OVT	d(CH₂)₅ ¹ , Tyr (Me) ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Tyr -NH ₂ ⁹	0.59	(21)
DTyrOVT	desGly-NH₂-d(CH₂)₅ ¹ , D-Tyr ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Gly -NH ₂ ⁹	0.94	*
DNalOVT	d(CH₂)₅ ¹ , D-2-Nal ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Tyr -NH ₂ ⁹	0.07	(21)
DThiOVT	d(CH₂)₅ ¹ , D-Thi ² , Ile ³ , Thr ⁴ , Asn ⁵ , Cys ⁶ , Pro ⁷ , Orn ⁸ , Tyr -NH ₂ ⁹	16.0	*

The ability of these peptides to promote inositol monophosphate (IP1) accumulation was first assayed by means of a homogeneous time-resolved FRET (HTRF) competitive immunoassay in which IP1 production is measured after a 30-min exposure to the different analogues used at a final concentration of 10 μM. As shown in Fig. 3, OT, AVP, AVT, Phe³OT, dLVT, Thr⁴OT, Thr⁴OVT, and dThr⁴OVT were all capable of inducing IP1 production, thus confirming their agonist proper-

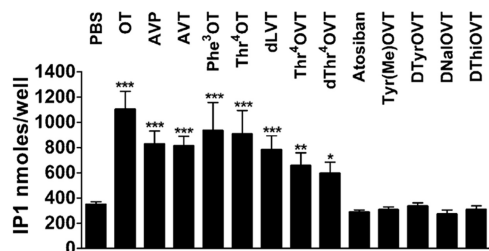


FIGURE 3. Inositol phosphate production in OTR-expressing HEK293 cells following OT and OT-derived peptide stimulation. IP1 production was measured using an immunocompetitive HTRF-based assay (HTRF IPOne, Cis-bio) in HEK293 cells stably expressing the N-terminally myc-tagged OTR (HEK mycOTR). A total of 100,000 cells were stimulated for 30 min with the OT and OT-derived peptides at a final concentration of 10 μ M. The data are expressed as the mean value $\pm$ S.D. (error bars), of three independent experiments, each performed in sextuplicate. One-way ANOVA followed by Dunnett's test was used to determine the statistical differences in IP1 production in the presence of the indicated ligand and untreated controls (PBS) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

ties in the G_q pathway, whereas atosiban, Tyr(Me)OVT, DTyrOVT, DNalOVT, and DThiOVT did not promote any significant IP1 production, confirming their previously reported antagonist properties in this pathway (20).

G_{α_q} Activation—As shown in Fig. 4a, incubation with OT, AVP, AVT, Phe³OT, dLVT, Thr⁴OT, Thr⁴OVT, and dThr⁴OVT significantly ($p < 0.001$) reduced energy transfer (BRET) between G_{α_q} and G_{γ_2} , reflecting the activation of the hOTR- $G_{\alpha_q}\beta\gamma$ complex. Atosiban, Tyr(Me)OVT, DTyrOVT, DNalOVT, and DThiOVT had no activating effect but did induce a modest but significant increase in energy transfer, indicating a distinct rearrangement of the trimeric G protein complex, the functional significance of which requires further investigation. Notably, there was a remarkable concordance between the BRET-based monitoring of G_q activation and the IP1 assay (Fig. 3), a finding that strongly validates this newly developed biosensor.

$G_{\alpha_{i1}}$ Activation—Fig. 4b shows that only three peptides (OT, AVT, and DNalOVT) significantly ($p < 0.001$) activated the hOTR- $G_{\alpha_{i1}}$ complex, as indicated by the decrease in the BRET signal. The OT and AVT peptides were also agonists of the OTR- G_q , - G_{i2} , and - G_{i3} complexes (Figs. 4, a, c, and d), whereas the BRET (Fig. 4a) and IP1 experiments (Fig. 3) showed that DNalOVT did not activate the OTR- G_q pathway or the OTR- G_{i2} , - G_{i3} , - G_{α_A} , and - G_{α_B} complexes (Fig. 4, c, d, e, and f). Dose-response experiments indicated that DNalOVT had an EC_{50} for $G_{\alpha_{i1}}$ activation of 38.83 ± 16.0 nM ($n = 3$) (Fig. 5a), very similar to the 62.63 ± 39.00 nM obtained using OT (Fig. 2). The fact that DNalOVT acted as an agonist of OTR- G_{i1} but not of the other screened OTR- G_{α} complexes identifies it as a functional selective $G_{\alpha_{i1}}$ agonist. Finally, as reported above in relation to G_q activation, some analogues induced a small increase in BRET energy transfer.

$G_{\alpha_{i2}}$ and $G_{\alpha_{i3}}$ Activation—Fig. 4, c and d, show that all of the peptides capable of inducing G_{α_q} activation also induced significant ($p < 0.001$) $G_{\alpha_{i2}}$ and $G_{\alpha_{i3}}$ subunit activation, as indicated by the decrease in the BRET ratio. Stimulation with Tyr(Me)OVT, DTyrOVT, DNalOVT, or DThiOVT had no effect on the variation in energy transfer efficiency in comparison with untreated cells.

Notably, atosiban induced a significant ($p < 0.05$) activation of the $G_{\alpha_{i3}}$ subunit, with a calculated EC_{50} of $2,800 \pm 1,035$ nM ($n = 3$) (Fig. 5b). Given that atosiban is characterized by a lower affinity for the human OTR than the other peptides used in this study (see Table 1), we monitored $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, $G_{\alpha_{\alpha_A}}$, and $G_{\alpha_{\alpha_B}}$ activation using higher atosiban concentrations (up to 1 mM) and confirmed that it had no effect on the other G_{α_i} and G_{α_o} complexes (supplemental Fig. 3). In conclusion, these data confirm the functional selective properties of atosiban and identify its selectivity for the OTR- G_{i3} complex. In this regard, it is important to note that the EC_{50}/K_i ratio of atosiban for the OTR- G_{i3} complex, 53, is in the same order of magnitude as that of OT, 14, which indicates a similar right shift in the EC_{50} value with respect to the apparent affinity of the two analogues.

$G_{\alpha_{\alpha_A}}$ and $G_{\alpha_{\alpha_B}}$ Activation—Fig. 4, e and f, show that only OT significantly activated ($p < 0.001$) the hOTR- $G_{\alpha_{\alpha_A}}$ and $G_{\alpha_{\alpha_B}}$ complexes, as indicated by the significant decrease in the BRET signal. All of the other peptides (including those active on $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, and $G_{\alpha_{i3}}$) were ineffective on these G_{α} subtypes, which proved to be remarkably selective for the OT-activated OTR.

β -Arrestin Recruitment and Receptor Internalization—The binding of an agonist to GPCRs leads to receptor activation, phosphorylation, and the translocation of β -arrestin to the receptor complex, an event that disrupts the receptor/G protein interaction and turns off the G protein-dependent signaling pathways. However, recent work has demonstrated that, in the case of a number of GPCRs, β -arrestins can mediate G protein-independent signaling by scaffolding cascade components, including small GTP-binding proteins and members of the MAPK family (29). The number of known biased ligands that can selectively activate β -arrestins without activating G protein signaling has rapidly increased over recent years (30), but only a few ligands that activate a G protein but do not promote β -arrestin recruitment have been described (30–32).

To investigate whether DNalOVT and atosiban induce β -arrestin recruitment, we used a “real-time” BRET¹ assay in which the OTR-RLuc construct acts as the energy donor, and the yellow variant of GFP (YFP) fused to the C terminus of β -arrestin1 and β -arrestin2 (β -arrestin1-YFP and β -arrestin2-YFP) acts as the acceptor (32) (Fig. 6). To compare the results obtained with the two different β -arrestins, we set up transfection conditions in which β -arrestins and OTR levels were maintained constant and comparable (see supplemental Fig. 1, c and d).

In cells co-expressing OTR-RLuc and β -arrestin1-YFP, OT used at a final concentration of 10 μ M increased the BRET ratio with a $t_{1/2}$ of 107 ± 31.25 s ($n = 3$); this remained stable for at least 10 min (Fig. 6a), thus indicating a rapid and sustained agonist-induced association between the OTR and β -arrestin1. Similar results were obtained using a β -arrestin2-YFP construct (Fig. 6b), whose $t_{1/2}$ of 18.37 ± 2.25 s ($n = 8$) confirmed the previously reported OTR/ β -arrestin2 kinetics (33). The specificity of these interactions was controlled using constructs in which RLuc and YFP were exchanged, and exactly the same results were obtained in cells expressing OTR-YFP and β -arrestin2-RLuc or RLuc- β -arrestin2 (supplemental Fig. 4). Fig. 6, c and d, shows the dose-response curves of OT-induced β -arrestin1 and β -arrestin2 recruitment, whose calculated EC_{50} values were 229 ± 23.15 and 41.15 ± 1.85 nM, respectively, thus indi-

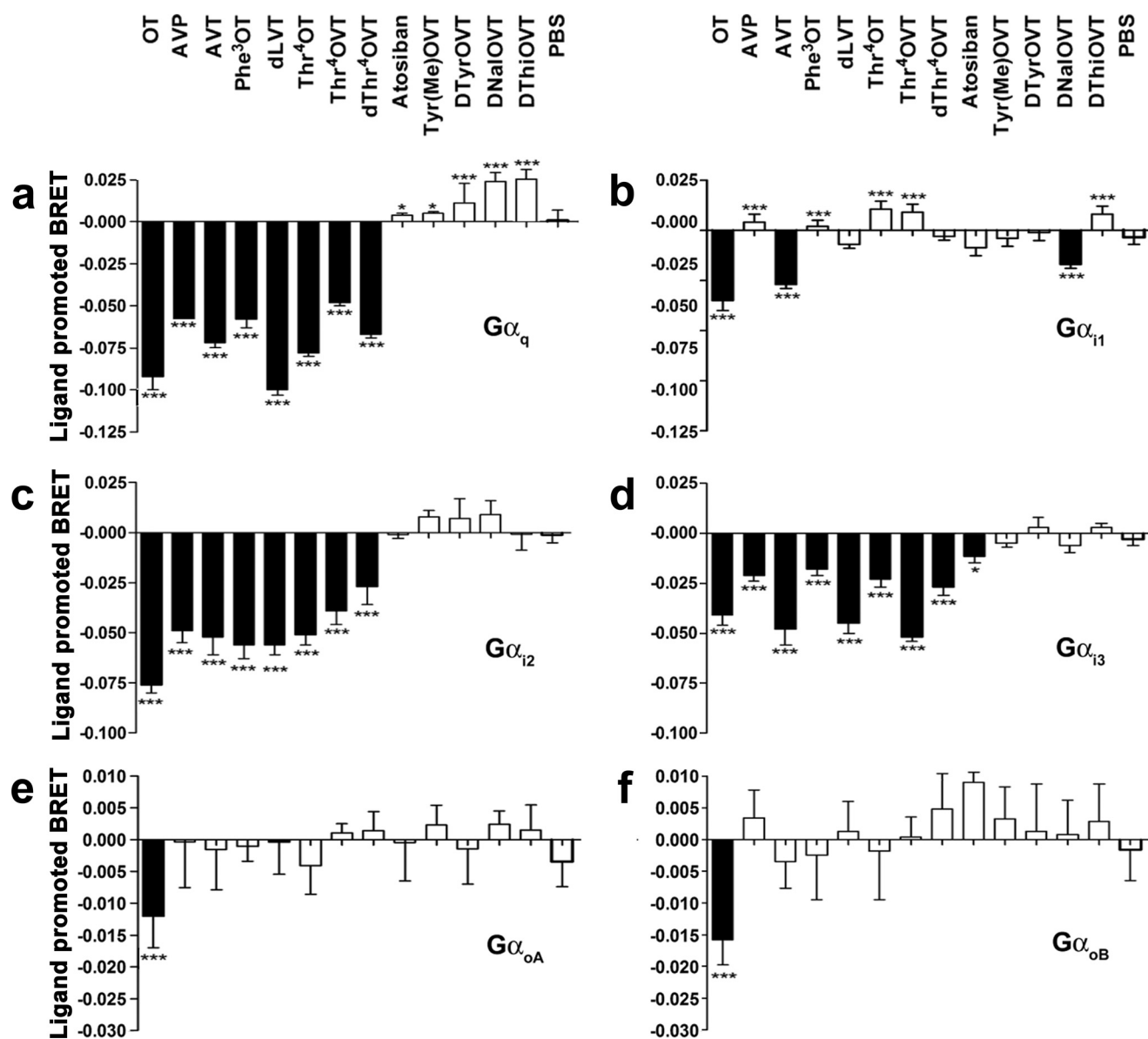


FIGURE 4. BRET measurements of OT and OT-derived ligand activation of OTR- $G\alpha\beta\gamma_2$ complexes in HEK293 cells co-expressing OTR, GFP¹⁰- $G\gamma_2$, $G\beta_1$, and the indicated $G\alpha$ Rluc constructs: $G\alpha_q$ -Rluc (a), $G\alpha_{i1}$ -Rluc (b), $G\alpha_{i2}$ -Rluc (c), $G\alpha_{i3}$ -Rluc (d), $G\alpha_{oA}$ -Rluc (e), and $G\alpha_{oB}$ -Rluc (f). The cells were stimulated for 2 min with OT and OT-derived peptides at a final concentration of 10 μ M; at this dose, OT produced a peak BRET ratio signal in all of the tested $G\alpha$ proteins. The results are the differences in the BRET signals in the presence and absence of ligands (10 μ M) and are expressed as the mean value $\pm$ S.D. (error bars) of at least six independent determinations. The statistical significance of the differences between stimulated and unstimulated (PBS) cells was assessed using one-way ANOVA followed by Dunnett's test (*, $p < 0.05$; ***, $p < 0.001$).

indicating that the OT-bound OTR has a higher affinity for β -arrestin2 than β -arrestin1. On the contrary, no association with β -arrestin1 or β -arrestin2 was found in the presence of atosiban (1 mM) or DNalOVT (10 μ M), as shown in Fig. 6, a and b.

We then investigated whether the $G\alpha_{i1}$ functional selective analog DNalOVT (which cannot recruit β -arrestins) is unable to promote ligand-induced receptor endocytosis, as we have previously shown for atosiban (8). HEK293 cells stably transfected with OTR-EGFP were incubated for 3 and 30 min with OT (100 nM), DNalOVT (100 nM), and atosiban (10 μ M), fixed, and observed using confocal microscopy. As shown in Fig. 6e, stimulation with OT led to the appearance of punctate fluorescence after only 3 min, which, after 30 min, had almost completely disappeared from the plasma membrane but continued

accumulating in the perinuclear region, thus indicating complete agonist-induced receptor internalization as reported previously (22). On the contrary, the fluorescence remained permanently localized at the cell surface after 30-min stimulation with atosiban or DNalOVT, and there was no receptor redistribution, indicating that neither DNalOVT nor atosiban induce receptor endocytosis.

DNalOVT Inhibits Cell Growth via a G_i -mediated Pathway—We finally tested the effect of DNalOVT on the proliferation of HEK293 cells stably expressing the OTR-enhanced green fluorescent protein (HEK OTR-EGFP) and DU145 human prostate cancer cells, which express endogenous OTR, because it has been shown that OT and atosiban inhibit cell growth in both lines via an OTR- $G\alpha_i$ -mediated pathway (8, 34) (Fig. 7). When

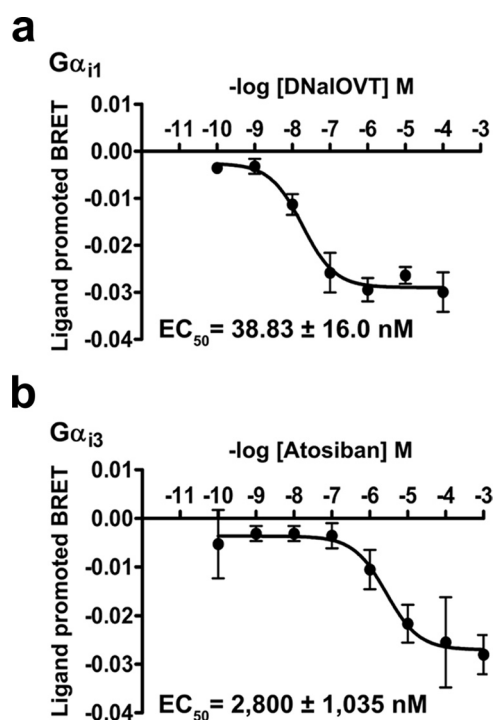


FIGURE 5. BRET measurements of DNaIOVT and atosiban dose-response activation of OTR- $G\alpha\beta_1\gamma_2$ complexes in HEK293 cells. The BRET ratio was recorded in HEK293 cells transfected with cDNAs encoding for OTR, GFP¹⁰, $G\gamma_2$, $G\beta_1$, and $G\alpha_{i1}$ -91-Rluc or $G\alpha_{i3}$ -91-Rluc for EC_{50} determination. The cells were stimulated with increasing concentrations of DNaIOVT (a) and atosiban (b) (from 10^{-10} to 10^{-3} M). The data are expressed as the mean value $\pm$ S.D. (error bars) of at least three independent determinations.

treated with OT, atosiban, and DNaIOVT (all used at a final concentration of 100 nM), HEK OTR-EGFP and DU145 cells both responded with a significant decrease in cell proliferation. The percentage of cell growth inhibition induced by OT, atosiban, and DNaIOVT was very similar in both cell lines (-18.6 ± 2 , -19 ± 7 , and $-15.1 \pm 1.9\%$ in HEK293; -27.9 ± 1.3 , -27 ± 0.2 , and $-24.1 \pm 0.7\%$ in DU145), which suggests that the three compounds have similar maximal efficacy. The inhibitory effects of DNaIOVT and atosiban on HEK293 cells were abolished by pretreatment with PTX ($+1.8 \pm 2.1\%$ and $+1.5 \pm 2\%$), thus supporting the involvement of a G_i -mediated pathway in cell growth inhibition (8). The effect of PTX could not be evaluated in DU145 cells because their slow doubling time requires a minimum of 72 h to observe cell growth inhibition, and this period of treatment with the toxin is itself cytotoxic and promotes cell death. Taken together, these results indicate that the independent activation of either G_{i1} (by DNaIOVT) or G_{i3} (by atosiban) can fully activate a signal transduction pathway, leading to cell growth inhibition in cells expressing a functionally G_i -coupled OTR.

DISCUSSION

We used a BRET-based approach for this first study of the ligand-promoted engagement of human OTRs with different G proteins. Our findings confirmed that OTRs recruit G_q and demonstrated that they interact with the three members of the G_i subfamily (G_{i1} , G_{i2} , and G_{i3}) and the two members of the G_o family, G_{oA} and G_{oB} . The use of newly developed biosensors to monitor receptor-induced G protein activation also showed

that human OTRs not only recruit but also activate G_q , G_{i1} , G_{i2} , G_{i3} , G_{oA} , and G_{oB} . Unlike an isolated previous study, in which very low amounts of $G\alpha_s$ associated with the OTR were identified by immunoadsorption (3), we did not find in HEK 293 cells any significant specific interaction between OTR and G_s , even in overexpression conditions. Moreover, the stimulation with OT did not induce the activation of the OTR- G_s complex. In our BRET-based assay, $G\alpha_i$ was activated by OT with an EC_{50} of 2.16 nM, which is the same as that obtained for the OT-induced accumulation of IP in human myometrial cells endogenously expressing the hOTR (1.4 nM; reported in Ref. 35) and in HEK293 cells transiently overexpressing the hOTR (1.7 nM; reported in Ref. 36). The finding of the same EC_{50} by means of BRET activation and IP measurements strongly validates the use of the G_q biosensor in determining OTR ligand efficacy.

The EC_{50} values of activation of the different G_i/G_o isoforms ranged from 11.5 nM (for $G\alpha_{i3}$) to 91.8 nM (for $G\alpha_{oB}$); the local concentration of the peptide, the level of expression of the individual isoforms, and their localization in specific plasma membrane domains with or without the receptor will thus all be important for determining the subunit-specific G_i/G_o coupling of endogenous OTR. Similarly, the at least 10-fold higher EC_{50} values of all of the $G\alpha_i$ and $G\alpha_o$ isoforms in comparison with $G\alpha_q$ indicates that G_i/G_o -mediated pathways are activated at higher OT concentrations than the G_q pathway. However, again, the outcome of the response *in vitro* and *in vivo* will depend on both the relative expression level and subcellular localization of the $G_q/G_{i/o}$ subunits and the local concentration of the peptide.

One important step toward identifying and functionally characterizing promiscuous OTR coupling is to gain insights into the molecular structure-function properties of different analogues. OT is a nonapeptide consisting of a cyclic core (residues 1–6) and a short terminal tripeptide (residues 7–9). Analysis of our data suggests that residues in the cyclic part of OT contribute to its remarkable broad capability to activate G_q , G_i , and G_o subtypes: (i) single substitution at residue 3 (as in Phe³OT) or 4 (as in Thr⁴OT and derived peptides) restricts the activation to G_q , G_{i2} , and $G\alpha_{i3}$, and (ii) the two compounds that showed exclusive $G\alpha_{i1}$ or $G\alpha_{i3}$ activation (DNaIOVT and atosiban) both present a bulky substitution at position 2, strongly supporting the role of this position in $G\alpha_i$ functional selectivity. Residues located in the terminal tripeptide seem to play a minor role in biased activity; an exception is represented by AVT, in which the substitution of Leu⁸ with a Tyr resulted in the specific loss of activity toward G_o subtypes; further analysis of peptides bearing a different substitution at position 8 will be necessary to fully address this point. In models of OT/OTR binding and activation, the terminal tripeptide interacts with the upper part of the first transmembrane helix (TM) and the second extracellular domain of the receptor and is critical for highly potent OT analogues, whereas the cyclic part extends more deeply into the transmembrane core and mediates receptor activation by interacting with a cluster of residues located in TM3, TM5, and TM6 (37–40). In particular, it has been suggested that the interaction of Tyr² of the peptide with a Phe located on TM6 promotes a change in the relative orientation of TM3 and TM6, breaks the intrahelix bond involving the arginine of the (E/D)RY motif,

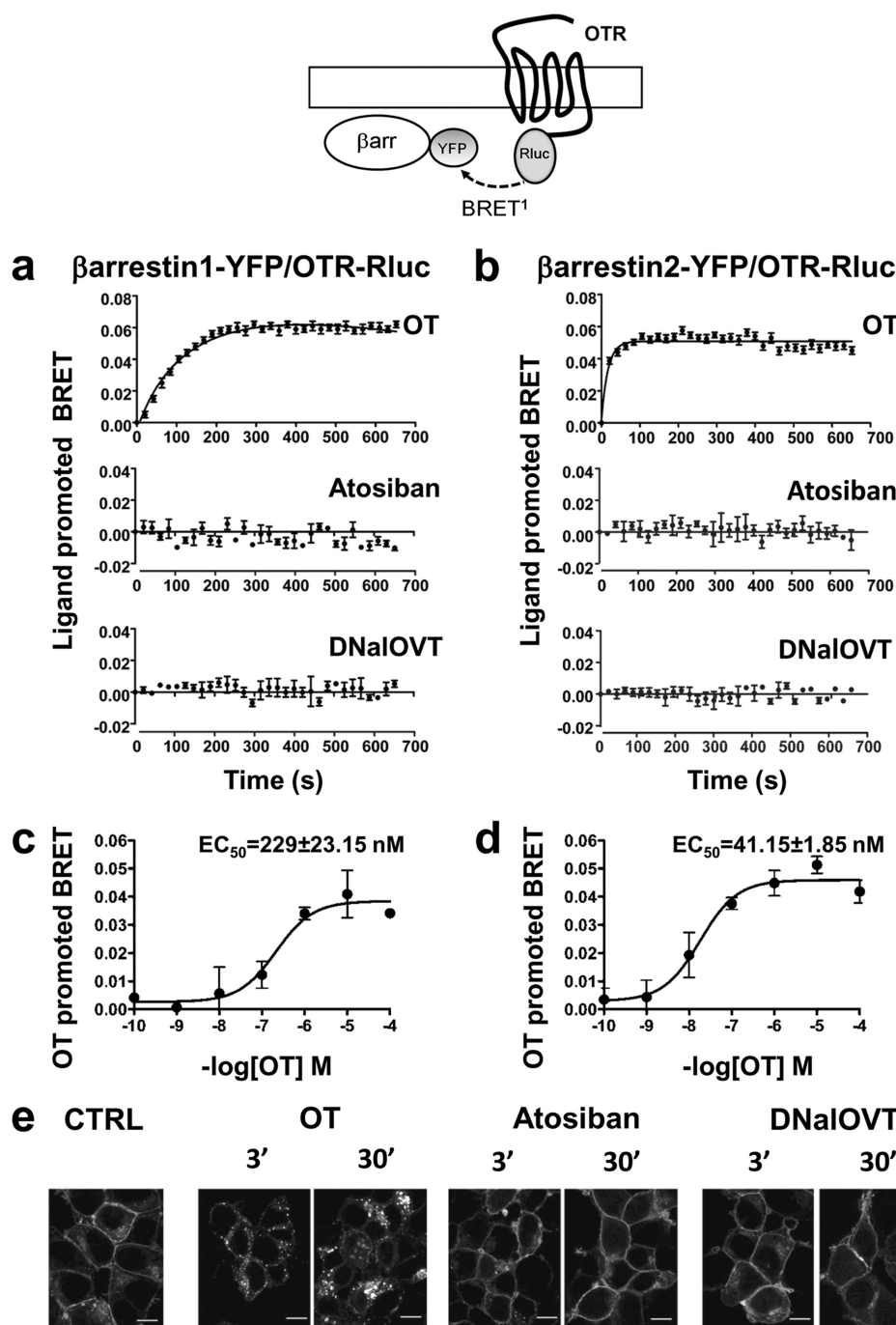


FIGURE 6. BRET measurements of OTR-mediated β -arrestin1 and β -arrestin2 recruitment in HEK293 cells following OT, atosiban, and DNalOVT stimulation. BRET¹ was monitored between Rluc and YFP introduced at the C-terminal of OTR (OTR-RLuc) and the β -arrestins: β -arrestin1-YFP (a) and β -arrestin2-YFP (b). HEK293 cells co-expressing OTR-RLuc and β -arrestins-YFP were stimulated by OT (10 μ M), DNalOVT (10 μ M), and atosiban (1 mM). Real-time BRET¹ measurements were made every 20 s. The results are the differences in the BRET signals in the presence and absence of agonist and are expressed as the mean value $\pm$ S.D. (error bars) of 3–5 independent experiments. c and d, BRET concentration-response curves of OT-induced β -arrestin recruitment in HEK293 cells. HEK293 cells co-expressing OTR-RLuc and β -arrestin1-YFP (c) or β -arrestin2-YFP (d) were treated with OT (10^{-10} to 10^{-4} M). The BRET signal was recorded at maximum plateau level (2 min for β -arrestin2 and 5 min for β -arrestin1). The results are the differences in the BRET signals in the presence and absence of agonist and are expressed as the mean value $\pm$ S.D. of three independent experiments. e, imaging of OTR-EGFP internalization upon ligand stimulation. The subcellular localization of recombinant OTR C-terminally fused to EGFP (OTR-EGFP) was visualized by means of laser scanning confocal microscopy in stably transfected HEK293 OTR-EGFP cells. The cells were fixed before (CTRL) and after incubation with OT (100 nM), DNalOVT (100 nM), and atosiban (10 μ M) for 3 and 30 min at 37 $^{\circ}$ C. Scale bar, 10 μ m.

and switches the receptor from an inactive to an active conformation (40); interestingly, the mutation in the Asp of the OTR (E/D)RY motif has also been shown to differentially affect G_q and G_i coupling (40). Our current data are consistent with the hypothesis that the chemical nature of the residue located at

this critical position will be crucial to determine the ability of peptidic ligands to induce/stabilize selective receptor active conformations.

A special set of agonist-induced GPCR conformations is represented by those leading to β -arrestin recruitment (41).

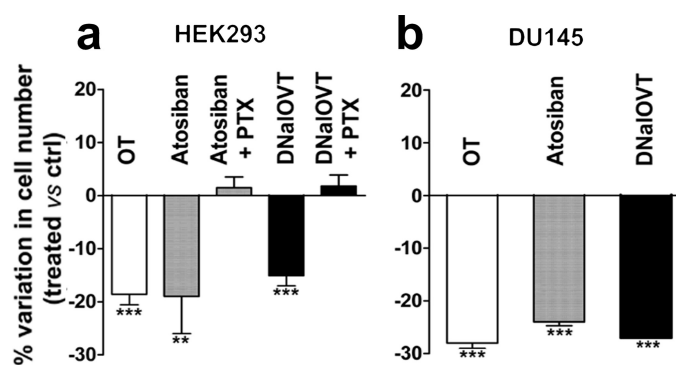


FIGURE 7. Atosiban and DNalOVT inhibit cell growth via a PTX-sensitive pathway. Atosiban and DNalOVT effects on cell growth were analyzed in HEK293 cells stably expressing the OTR fused to EGFP (OTR-EGFP) (a) and DU145 human prostate cancer cells endogenously expressing OTR (b). The cells were treated with OT, DNalOVT, and atosiban at a final concentration of 100 nM in the presence or absence of PTX (150 ng/ml). Cell growth was determined by means of an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS)-based assay after 48 h (HEK293 cells) or 72 h (DU145 cells) of peptide treatment. The results are the percentage variations in the number of cells under treated versus untreated conditions (ctrl); the differences were statistically analyzed using one-way ANOVA followed by Dunnett's test (**, $p < 0.01$; ***, $p < 0.001$). Error bars, S.D.

Ligands that specifically recruit β -arrestins in the absence of G protein activation have been described for various GPCRs, including serotonin, opioid, vasopressin, dopamine, and β -adrenergic receptors (42). This allows the identification of β -arrestin-mediated signaling mechanisms promoted by selective receptor conformations. However, it is not known whether OTR coupling to different G proteins differentially affects β -arrestin recruitment and/or internalization. Upon OT activation, OTRs are phosphorylated by GRK2, bind β -arrestin, and are endocytosed via clathrin-coated vesicles (33, 42, 43); after internalization, they recycle back to the plasma membrane via the Rab4/Rab5 short recycling pathway (22). Because neither atosiban nor DNalOVT promoted β -arrestin1 or β -arrestin2 recruitment and receptor internalization, we suggest that active OTR conformations coupling to G_i do not efficiently recruit β -arrestins, which would be in line with published data showing that the recruitment of β -arrestins is also $G\alpha_i$ -independent in prostaglandin E2 receptors (44) and protease-activated receptor 1 (45, 46). Whether these active conformations correspond to phosphorylated or unphosphorylated forms remains to be established.

Taken together, these data support the idea that, within a given GPCR, different ligands trigger/stabilize different G protein-specific active conformations. In the case of OTRs, the endogenous OT ligand exquisitely evolved to activate not only G_q but all members of the G_i and G_o families. None of the other peptides tested in this screening (which included AVP, the other endogenous and closely related neurohypophyseal peptide) showed such an extended degree of G protein subtype activation. One interesting finding is that all of the peptides activating G_q also activated G_{i2} and G_{i3} , but none of them efficiently engaged G_{oA} or G_{oB} , and only AVT activated G_{i1} . Even more interestingly, DNalOVT and atosiban only activated a single G_i subtype, $G\alpha_{i1}$ and $G\alpha_{i3}$, respectively. These findings together indicate that ligands can discriminate different G_i

family members at a single GPCR. G_i/G_o -biased activity has been reported previously (47, 48), but, to the best of our knowledge, this is the first clear example of ligands biased toward a single G_i family member.

Our findings open up a way for the development and use of functionally selective peptides acting on different $G\alpha_i$ -mediated pathways. Knowing the receptor-specific coupling to G_i/G_o subunits is particularly important because they are different in terms of tissue distribution and have only partially overlapping functions (49). $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_{i1}$ are primarily found in the nervous system, whereas $G\alpha_{i2}$ is ubiquitously expressed and is the quantitatively predominant $G\alpha_i$ isoform; $G\alpha_{i3}$ is hardly detectable at the protein level in the neuronal system but is widely expressed in peripheral tissues (49). The G_i effectors include adenylyl cyclase inhibition and ion channel modulation, whereas the neuronal effects of G_o seem to be almost exclusively mediated by its activity on ionic conductances (49); finally, G_o isoforms couple multiple receptors to calcium channels, whereas coupling to potassium channels preferentially requires G_i subunits (49). As OTRs are expressed in various peripheral tissues and organs, as well as in various brain regions, they may couple to G_q and different $G\alpha_i$ and $G\alpha_o$ isoforms, thus leading to the activation of different effectors. Although their use in humans is hampered by their agonist activity on the related V1a vasopressin receptor subtype (21), atosiban and DNalOVT can be instrumental in identifying the role played by promiscuous OTR coupling in eliciting various OT-mediated neuroendocrine and behavioral effects. It has been previously shown that atosiban (which our current results indicate only activates the peripherally expressed G_{i3} subunit) inhibits the growth of mammary and prostate cancer cells *in vitro* and *in vivo* (10) and may act as a leading peptide to guide the development of G_{i3} -selective analogues that may help control proliferative disorders. It is worth mentioning here that activating G_{i3} alone (using atosiban) is sufficient to inhibit cell growth, so unwanted effects mediated by other $G_{i/o}$ family members can be avoided. Furthermore, because OT plays a pivotal role in the CNS and shows promise in autism and schizophrenia (11–12), it is of paramount importance to define the role played by OTR differential coupling in regulating different social and cognitive behaviors. We have recently demonstrated that in neuronal cells, OTR activation has a dual role on neuronal excitability: inhibiting inward rectifying conductances via a pertussis toxin-resistant G protein and phospholipase C pathway and activating inward rectifying current via receptor coupling to a pertussis toxin-sensitive $G_{i/o}$ protein (4). Thus, the functional selective OTR- G_{i1} analog DNalOVT may be particularly helpful in identifying selective OTR- G_{i1} -mediated functions in the brain.

Finally, because these G_i functional selective ligands activate OTRs without inducing receptor internalization, it would be very interesting to investigate whether the absence of β -arrestin recruitment to the receptor, which is generally associated with desensitization, could result in longer lasting response. Whether the lack of β -arrestin recruitment could also lead to the loss of a specific signaling pathway that requires β -arrestin

engagement (41) also remains to be investigated. Such ligand-biased signaling may have important implications for the *in vivo* effects of drugs targeting OTR and may contribute to the discovery of compounds with unique pharmacological properties that may lead to the development of drugs with better therapeutic profiles.

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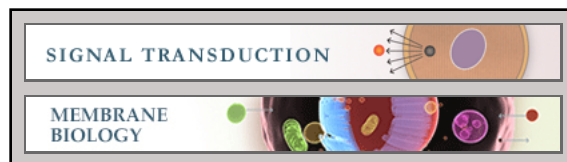
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