We used a bioluminescence resonance energy transfer bio-sensor 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_{12} and G_{13}, but not G_{11}, G_{oA}, or G_{oB}, indicating a strong bias toward these subunits. Two peptides (DNAoVT and atosiban) activated only G_{11} or G_{10} 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) 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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2. This article contains supplemental Figs. 1–4.

3. The abbreviations used are: GPCR, G protein-coupled receptor; AVP, arginine vasopressin; BRET, bioluminescence resonance energy transfer (BRET uses RLuc as a donor, the h derivative of coelenterazine as its substrate, and YFP as the acceptor; BRET' uses RLC as a donor, the coelenterazine 400a coelenterazine derivative as its substrate, and GFP as a target); Gα_{nA}, a G protein α subunit subtype (Gα_{nA}, Gα_{1A}, etc.); Gα_{nB}, β γ subunit subtype (Gγ_{1A}, Gγ_{1B}, etc.); Gβ_{1A}, a heterotrimeric complex containing α_{1A} as the α subunit (e.g., Gβ_{1A} as a heterotrimeric complex containing α_{2} as the α subunit); Gγ_{1A}, 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; PTK, protein tyrosine kinase; RR, Renilla luciferase; RLUC8, a Renilla luciferase mutant with a total of eight favorable mutations; V1a, vasopressin receptor subtype 1a; V1b, vasopressin receptor subtype 2b; TM, transmembrane helix; ANOVA, analysis of variance; AVT, arginine vasotocin.
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“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 Gq and Gi heterotrimeric complexes has been described in several cell systems (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 Goq,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 Gi is responsible for inhibiting cell growth, whereas receptor coupling to a pertussis toxin (PTX)-insensitive complex (possibly Gq) stimulates cell growth (5, 7).

Because of the 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-Gi 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 Gi0 over Gq 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-Gi 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 Gq, Gi0, Gi1, Gi2, Gi3, GoA, GoB, and Gc-transducing complexes.

We found that all of the tested AVP and OT analogues harboring substitution in functionally important domains of the peptides activate Gq, Gi2, and Gi3 with comparable relative efficacy, but none of them (not even those that activate the other Gi members as effectively as OT) can reliably activate Gi0, GoA, and GoB, thus indicating a bias toward these subunits; furthermore, two compounds (DNalOVT and atosiban) were entirely biased toward Gi1 or Gi3 activation, representing the first examples of biased ligands differentiating Gi1,2 subtypes. We also found that the Gi 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 Goq,97-Rluc, Goi1,91-Rluc, Goi2,91-Rluc, Goi3,91-Rluc, Gaoa,91-Rluc8, GaoA,91-Rluc8, and GoB,113-Rluc8 cDNAs are described elsewhere.4 The Goq, Gi1, Gi2, Gi3, GaoA, GaoB, and Gγ2 cDNAs came from the Missouri S&T cDNA Research Center (Rolla, MO). The plasmids encoding GpF10-Gγ2 and Gβ5 have been described previously (13); the plasmids encoding for the human OTR and V1R are described in Refs. 14 and 15; and OTR-Rluc, OTR-YFP, and OTR-EGFP are as described in Refs. 7, 10, 16, and 17. GpF10-Rluc 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′-CACAAAAGCTTATGGAGGGCGCGCTCGCAG-3′) and reverse (5′-GTGGATCCCGTGGATGGCTGGGAGCAG-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 Wuerzburg). 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 described 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-termi-

nally 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. Altie´, M. H. Seguelas, J. L. Hansen, J. M. Sénard, and C. Galés, 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 polyethyleneimine (PEI linear, M, 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 further 24 h before the experiments. 48 h after transfection, the cells were washed twice, detached, and resuspended with PBS, 0.5 mM MgCl₂ 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 (Kᵢ) 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 x 10⁻⁹ M. Nonspecific binding was determined in the presence of unlabeled OT (10⁻⁷ M). The ligand binding data (Kᵢ) 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 Gα subunits by means of BRET² experiments, HEK293 cells were co-transfected with OTR-Rluc, GFP²-Gₐ₂₆, β₂₅, and one of Gα₋₁1, Gα₋₁₃, Gα₋₁₆, Gα₇, Gα₁₆, Gα₁₉, or Gα₂₃. To screen for the effects of the different ligands on G protein activation, HEK293 cells were transiently transfected with Gα₋₁1-Rluc, Gα₋₁₃-Rluc, Gα₋₁₆-Rluc, Gα₁₆-Rluc, Gα₁₉-Rluc, Gα₁₉-91-Rluc8, Gα₂₃-91-Rluc8, and Gα₋₁₁₃-Rluc8 constructs in the presence of plasmids encoding for GFP¹₀-Gₐ₂₆, Gβ₁₂, and the OTR or V₂,R. Finally, to study OTR-mediated β-arrestin recruitment by means of BRET³ experiments, the cells were co-transfected with OTR-Rluc and β-arrestin1-YFP or β-arrestin2-YFP or with OTR-Rluc 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 MgCl₂ 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,

\[
\text{Ligand-promoted BRET} = \frac{\text{emission GFP}^{10}_{\text{signal}}/\text{emission Rluc}_{\text{signal}}}{\text{emission GFP}^{10}_{\text{PBS}}/\text{emission Rluc}_{\text{PBS}}} \quad \text{(Eq. 1)}
\]

For titration experiments, HEK293 cells were transfected using a constant amount of Gα₋₁₁₃-Rluc8 with increasing amounts of OTR-GFP⁰ 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 BRET² 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 (t½) 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% CO₂). The stimulation was blocked by putting the dishes on ice and washing the cells three times with ice-cold PBS/Ca²⁺/Mg²⁺ (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.1 mM CaCl₂, 1 mM MgCl₂, pH 7.4).
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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 ± 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 Gaₒ₁, Gaₒ₂, and Gaₓ₁ Subunits—To investigate the coupling specificity of human OTs, 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 Gγ₂ subunit N-terminally fused with a blue-shifted variant of Aequeorea victoria green fluorescent protein (GFP₁₀-GÔ), and one of the seven different Ga subunits (Gaₒ₁, Gaₒ₂, Gaₒ₃, Gaₒ₄, Gaₒ₅, Gaₒ₆, or Gaₒ₇) (25). The cells were then stimulated for 2 min with 10 μM 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 Gaₒ₁, Gaₒ₂, Gaₒ₃, Gaₒ₄, Gaₒ₅, and Gaₒ₆ subunits (Gaₒ₇ was demonstrated to have no effect on the BRET signal). This interaction of the OTR with the Gγ₂ and Ga subunits; no statistically significant increase in the BRET signal was observed after co-expression of the Gaₒ subunit.

Because previous work in the literature reported OTR-Gaₒ 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 and of a negative control CD4-GFP¹⁰ over a fixed amount of Gaₒ₋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 Gaₓ₋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 Ga and Gγ subunits of the heterotrimeric G-protein complex accurately measures the separation of the Ga and Gβγ subunits that follows receptor activation (13). The energy donor (Rluc) is inserted within the Ga subunit amino acid sequence, and the acceptor (GFP¹⁰) is N-terminally fused to the Gγ₂ subunit (GFP²⁻Gγ₂). Previously engineered to measure Gaₒ₁ activation (13), BRET probes have now been built for all of the Ga protein isoforms.⁴

An Gaₓ₋₁₁₃-Rluc8 was first used to confirm that OT is unable to induce OTR-Ga 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 Gaₒ₁, Gaₒ₂, Gaₒ₃, Gaₒ₅, Gaₒ₆, and Gaₒ₇ with the Gaₒ₋₉₇-Rluc, Gaₒ₋₁₁₃-Rluc, Gaₒ₋₁₁₃-Rluc, Gaₒ₋₁₁₃-Rluc, Gaₒ₋₁₁₃-Rluc, Gaₒ₋₁₁₃-Rluc, and Gaₒ₋₁₁₃-Rluc constructs; Rluc8 (27) constructs were used to characterize Gaₒ₁ and Gaₒ₇ functional selectivity because the agonist-promoted BRET variation was more pronounced and less variable with them than with the Rluc. 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 Ga-Rluc constructs (mean 24,390 ± 841.5 arbitrary units). In the case of Ga-Rluc8 constructs, 4-fold higher values were obtained (104,300 ± 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 Ga subunits are comparable.

Using these probes and experimental conditions, we found that OTs not only recruit but also activate Gaₒ₁, Gaₒ₂, Gaₒ₃, Gaₒ₅, Gaₒ₆, and Gaₒ₇, 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 Gaₓ subunit-transfected cells.

Screening of Functional Selective Ligands; Identification of Functional Selective Gaₒ₁ and Gaₒ₃ 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-Gαβγ 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 Gβγ subunit (GFP¹⁰-Gβγ). OT-induced OTR-Gα coupling places OTR-Rluc and GFP¹⁰-Gβγ 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-Gβγ, and Gβγ in the absence (−α, empty bar) or presence of the indicated Gα subunits ([αq, α1q, α1q, α12, α13, ααA, ααB, αs]). The results are the differences in the BRET signal with OT (10⁻⁶ M) 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 Gα₁₁₃-Rluc8 and OTR-GFP² or CD4-GFP¹⁰ in combination with b2 Gβγ subunits. The amount of plasmid encoding GFP²-tagged proteins varied (from 0.031 to 8 μg), whereas the amount of Gα₁₁₃-Rluc8 was kept constant (3 μg). Data are representative of two experiments and were fit using linear regression. c, Gα₁ activation was evaluated with BRET in HEK293 cells co-expressing OTR or the positive control V₂R with GFP¹⁰-Gβγ, Gβγ, and Gα₁-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 Gβγ (GFP¹⁰-Gβγ), 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-Gβγ, Gβγ, and Rluc/Rluc8-tagged Gα subunits: αo (n = 6), αq (n = 14), ααA (n = 5), ααB (n = 8), ααA (n = 3), and ααB (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).

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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), Kᵢ 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 subtypes (14, 20). Second, to identify the residue(s) that contribute to converting the unselective Gαq/Gαo endogenous ligand OT into the functional selective Gαq/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 separately replaced by D-Tyr(Et) to obtain the Biased Analogs at Individual G Protein Family Subtypes.

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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), Kᵢ 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 subtypes (14, 20). Second, to identify the residue(s) that contribute to converting the unselective Gαq/Gαo endogenous ligand OT into the functional selective Gαq/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 separately replaced by D-Tyr(Et) to obtain the Biased Analogs at Individual G Protein Family Subtypes.
The ability of these peptides to promote inositol monophosphate (IP1) accumulation was first assayed by means of a homogenous 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-
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Notably, atosiban induced a significant \((p < 0.05)\) activation of the \(G_{\alpha_{i3}}\) subunit, with a calculated EC\(_{50}\) of 2,800 ± 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 \(G_{\alpha_{i1}}, G_{\alpha_{i2}}, G_{\alpha_{i3}}, G_{\alpha_{oA}}, \) and \(G_{\alpha_{oB}}\) activation using higher atosiban concentrations (up to 1 mM) and confirmed that it had no effect on the other \(G_{\alpha}\) and \(G_{\alpha_{o}}\) complexes (supplemental Fig. 3). In conclusion, these data confirm the functional selective properties of atosiban and identify its selectivity for the OTR-\(G_{\alpha_{i3}}\) complex. In this regard, it is important to note that the EC\(_{50}/K_i\) ratio of atosiban for the OTR-\(G_{\alpha_{i3}}\) 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.

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**FIGURE 3.** Inositol phosphate production in OTR-expressing HEK293 cells following OT and OT-derived peptide stimulation. IP\(_1\) production was measured using an immunocompetitive HTRF-based assay (HTRD IPOne, Cisbio) 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 μM. The data are expressed as the mean value ± 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 IP\(_1\) production in the presence of the indicated ligand and untreated controls (PBS) \(*, p < 0.05; **, p < 0.01; ***p < 0.001)."
cating 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 μM), as shown in Fig. 6, a and b.

We then investigated whether the Gα1i 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 2 and 30 min with OT and OT-derived peptides at a final concentration of 10 μM; at this dose, OT produced a peak BRET ratio signal in all of the tested Gα proteins. The results are the differences in the BRET signals in the presence and absence of ligands (10 μM) and are expressed as the mean value ± 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 < 0.01; ***, p < 0.001).

DNalOVT Inhibits Cell Growth via a Gαi-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-Gα1i-mediated pathway (8, 34) (Fig. 7). When
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that human OTRs not only recruit but also activate \( G_q, G_{11}, G_{12}, G_{13}, G_{oA}, \) and \( G_{oB}. \) Unlike an isolated previous study, in which very low amounts of \( G_\alpha \) associated with the OTR were identified by immunoabsorption (3), we did not find in HEK 293 cells any significant specific interaction between OTR and \( G_\alpha \) even in overexpression conditions. Moreover, the stimulation with OT did not induce the activation of the OTR-\( G_\alpha \) complex. In our BRETr-based assay, \( G_\alpha \) was activated by OT with an EC\textsubscript{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\textsubscript{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\textsubscript{50} values of activation of the different \( G_i/G_o \) isoforms ranged from 11.5 nM (for \( G_{oA} \)) to 91.8 nM (for \( G_{oB} \)); 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\textsubscript{50} values of all of the \( G_o \) and \( G_\alpha \) isoforms in comparison with \( G_i \) indicates that \( G_i/G_\alpha \)-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 \( G_q/G_i/G_o \) 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 Phe\textsubscript{OT}) or 4 (as in Thr\textsubscript{4OT} and derived peptides) restricts the activation to \( G_{i1}, \) \( G_{oA}, \) and \( G_{oB} \), whereas the cyclic part extends more deeply into the intrahelix bond involving the arginine of the (E/D)RY motif, thereby facilitating its interaction with \( G_{i1}, \) \( G_{oA}, \) and \( G_{oB} \), and neutralizing the effect of Tyr\textsubscript{2} 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.
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 Gq and Gı 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 6. BRET measurements of OTR-mediated β-arrestin1 and β-arrestin2 recruitment in HEK293 cells following OT, atosiban, and DNalOVT stimulation. BRET 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 μM), DNalOVT (10 μM), and atosiban (1 mM). Real-time BRET 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 ± 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 10⁻⁶ 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 ± S.D. of three independent experiments. e, imaging of OTR-GFP 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 μM) for 3 and 30 min at 37 °C. Scale bar, 10 μm.
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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 Gq do not efficiently recruit β-arrestins, which would be in line with published data showing that the recruitment of β-arrestins is also Gαi-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 Gq but all members of the Gi and Gq families. None of the other peptides tested in this screening (which included AVP, the other endogenous and closely related neurohypophysial peptide) showed such an extended degree of G protein subtype activation. One interesting finding is that all of the peptides activating Gq also activated Gi2 and Gi3, but none of them efficiently engaged Gai or Goi0 and only AVT activated Gi3. Even more interestingly, DNalOVT and atosiban only activated a single Gi subtype, Gai1 and Gai3 respectively. These findings together indicate that ligands can discriminate different Gq family members at a single GPCR. Gq/Gai-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 Gq family member.

Our findings open up a way for the development and use of functionally selective peptides acting on different Gαq-mediated pathways. Knowing the receptor-specific coupling to Gq/Gai subunits is particularly important because they are different in terms of tissue distribution and have only partially overlapping functions (49). Gαq11, Gαq16, and Gai1 are primarily found in the nervous system, whereas Gai3 is ubiquitously expressed and is the quantitatively predominant Gai isoform; Gai3 is hardly detectable at the protein level in the neuronal system but is widely expressed in peripheral tissues (49). The Gq effectors include adenylate cyclase inhibition and ion channel modulation, whereas the neuronal effects of Gai seem to be almost exclusively mediated by its activity on ionic conductances (49); finally, Gα, isoforms couple multiple receptors to calcium channels, whereas coupling to potassium channels preferentially requires Gq subunits (49). As OTRs are expressed in various peripheral tissues and organs, as well as in various brain regions, they may couple to Gq and different Gai and Gqi 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 Gai3 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 Gai3-selective analogues that may help control proliferative disorders. It is worth mentioning here that activating Gai3 alone (using atosiban) is sufficient to inhibit cell growth, so unwanted effects mediated by other Gai/Go 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 Gai0 protein (4). Thus, the functional selective OTR-Gai analog DNalOVT may be particularly helpful in identifying selective OTR-Gai-mediated functions in the brain.

Finally, because these Gq 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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