Molecular Pharmacology Fast Forward. Published on November 22, 2011 as doi:10.1124/mol.111.074757

MOL #74757

Lateral allosterism in the glucagon receptor family: GLP-1 induces GPCR heteromer formation

Dominik Schelshorn, Fanny Joly, Sophie Mutel, Cornelia Hampe, Billy Breton, Vincent Mutel, Robert Lütjens

(Addex Pharmaceuticals, Chemin des Aulx 12-14, CH-1227 Plan-les-Ouates, Geneva, Switzerland)

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

GLP-1 binding induces heteromerization of GLP-1R and GIPR

Corresponding author: Dominik Schelshorn (Addex Pharmaceuticals, Chemin des Aulx 12-14, CH-1227 Plan-les-Ouates, Geneva, Switzerland. Tel: +41-22-8841555, Fax: +41-22-8841556. Email: dominik.schelshorn@addexpharma.com

Number of text pages: 35

Tables: 1

Figures: 9

Supplementary Figures: 3

References: 39

Word count:

Abstract: 243

Introduction: **662**

Discussion: 1500

Abbreviations:

$A_{2A}R$ = adenosine receptor 2a

AA = amino acid

BRET = bioluminescence resonance energy transfer

D1R/D2R/D3R = dopamine receptor 1/2/3

ECD = extracellular domain

ER = endoplasmatic reticulum

FRET = fluorescence resonance energy transfer

GIPR = gastric inhibitory protein receptor

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

GlucR = glucagon receptor

GLP-1R = glucagon-like peptide-1 receptor

GLP-2R = glucagon-like peptide-2 receptor

mGluR = metabotropic glutamate receptor

M4R = muscarinic acetylcholine receptor

MTR1/MTR2 = melatonin receptor 1/2

PKA/PKC= protein kinase A/C

PTHR = parathyroid hormone receptor

RAMP= receptor activity modifying proteins

$T1R_1/T1R_2/T1R_3$ = sweet and umami taste receptor 1/2/3

V1b-R= vasopressin V1b receptor

VPAC-R= vasoactive intestinal polypeptide receptor

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Activation of a G-protein coupled receptor (GPCR) results in a variety of cellular responses, for example, through binding of different ligands to the same receptor that activate distinct downstream cascades. Additional signaling complexity is achieved when two or more receptors are integrated into one signaling unit. Lateral receptor interactions can allosterically modulate the receptor response to a ligand thereby create a mechanism of tissue specific fine tuning, depending on the cellular receptor co-expression pattern. GPCR homo- or heteromers have been explored widely in GPCR class A and C, but to lesser extent in class B. In the present study, we used Bioluminescence Resonance Energy Transfer (BRET) techniques, calcium flux measurements, and microscopy to study receptor interactions within the glucagon receptor family. We found basal BRET interactions in some of the receptor combinations tested that decreased upon ligand binding. A BRET increase was exclusively observed between the gastric-inhibitory-peptide-receptor (GIPR) and the glucagon-like-peptide-1-receptor (GLP-1R) upon binding of GLP-1, and could be reversed by GIP addition. The interactions of GLP-1R and GIPR were characterized by BRET-donor saturation studies, shift experiments, and testing of glucagon-like ligands. The heteromer displayed a specific pharmacology in GLP-1 induced β-arrestin recruitment and calcium flux, suggesting a form of allosteric regulation between the receptors. This study provides the first example of ligand-induced heteromer formation in GPCR class B. In the body, both receptors are functionally related and co-expressed in the same cells. The physiological evidence of this heteromerization remains to be determined.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

G-protein coupled receptors (GPCR) are integral plasma membrane proteins that trigger cellular responses to stimuli as diverse as light, smell, taste, hormones, and neurotransmitters. GPCRs is one of the largest protein families in the genome and in vertebrates the main sub-classes are formed by the rhodopsin-like receptors (class A), the secretin-like receptors (class B), and the glutamate-like receptors (class C). GPCRs activate heterotrimeric G-protein signalling in the cytoplasm but additional receptor interactions with kinases, arrestins, molecular chaperones, RAMPs, and PDZ-domain containing proteins (Bockaert et al., 2004) also contribute to the specific cellular response.

GPCRs laterally interact with each other in the plasma membrane (Bouvier, 2001). Self-association (homomerization) and association with other receptors (heteromerization) can form dimers, trimers, or oligomers of higher order. The receptors in such complexes are thought to allosterically modulate each other, creating a new receptor type with unique pharmacology (Smith and Milligan, 2010). FRET and BRET techniques have been used extensively to monitor real time interactions between GPCRs in living cells (Pfleger and Eidne, 2005). Data on receptor-receptor interactions have been collected in a GPCR oligomerization database, some of the described interactions were confirmed to be physiologically relevant and exist in vivo (http://data.gpcr-okb.org/gpcr-okb/). Receptor dimers are believed to exist for many GPCRs and may couple to trimeric G-proteins in a 2:1 stoichometry for a minimal functional unit (Han et al., 2009).

Class A heteromers Constitutive heteromers can exist independent of ligand binding and remain associated throughout the lifecycle of a receptor. One of many examples in the class A are the dopamine receptor heteromers D1R/D2R and D1R/D3R, which

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

exist in the rodent brain and display a distinct pharmacology (Marcellino et al., 2008;Rashid et al., 2007). Heteromer formation upon ligand binding has been reported for example for D2R/SSTR5 (Rocheville et al., 2000) and SSTR1/SSTR5 (Patel et al., 2002). In other cases, ligand-induced increases of the energy transfer in the BRET experiment has been interpreted as a movement within an existing heteromer, for example, for MTR1/MTR2 (Ayoub et al., 2002).

Class C heteromers In class C, most of the metabotropic glutamate receptors (mGluRs) are known to form heterodimers (Doumazane et al., 2011), whereas the $Gaba_{B1/B2}$ receptors form dimers or tetramers (Pinard et al., 2010) that undergo a dissociation movement upon ligand binding (Matsushita et al., 2010). Other heteromers include the sweet and umami taste receptors $T1R_{1/2}$- and $T1R_3$ (Li et al., 2002), and the discovery of an mGluR5/D2R/A_{2A}R heteromer provided evidence of class A/C mixed heteromers (Cabello et al., 2009).

Class B heteromers CRF-R1 has been shown to interact with V1b-R (Young et al., 2007), while the secretin receptor (SecR) forms heteromers with VPAC-R1 and VPAC-R2 (Harikumar et al., 2006), and other closely related receptors of which only the SecR/PTHR heteromer showed a decreasing BRET signal upon ligand binding (Harikumar et al., 2008). VPAC receptors have also been found to form heteromers that on ligand binding exhibited reduced BRET signals. Interestingly, none of the above mentioned studies observed ligand-induced heteromer recruitment for class B GPCRs.

Co-expression in the same cell is mandatory for a physiological relevance of receptor heteromerization. Lateral regulation of receptor function in such heteromers may occur between receptors that are clustered in the same physiological function. The members of the glucagon receptor family; the glucagon receptor (GlucR), the

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

glucagon-like peptide -1 and -2 receptors (GLP-1R, GLP-2R), and the gastric inhibitory protein receptor (GIPR) regulate glucose homeostasis and fulfill those criteria. Co-expression has been demonstrated, for example, in pancreatic α-cells, thalamus and hypothalamus (GlucR and GLP-1R), pancreatic β-cells and heart (GlucR, GLP-1R, GIPR), stomach (GLP-1R, GLP-2R), the cerebral cortex and hippocampus (GlucR, GIPR) (Mayo et al., 2003;Brubaker and Drucker, 2002;Alvarez et al., 2005).

In the present study, we assessed heteromeric interactions in the glucagon receptor family. Fusing GlucR, GLP-1R, GLP-2R, and GIP-R to the luciferase RLuc8 or the fluorescent protein YPet, we measured possible interactions by BRET (Kamal et al., 2009). Ligand-induced heteromer formation was profiled pharmacologically and effects on receptor internalization, arrestin recruitment, and calcium flux were tested.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials: Ligands: (h=human, brackets = amino acid positions) hGlucagon (1-29), hOxyntomodulin, hGLP-1(7-36)NH_2, (all Bachem AG, Bubendorf, Switzerland), hGLP-2(146-178), hGIP(1-42) (both Anaspec, Fremton, CA, USA), hGLP-1(9-36), Exendin(9-39), Exendin 4 (all Tocris Bioscience, Bristol, UK) and (Pro^3)GIP (Phoenix Europe GmbH, Karlsruhe, Germany) were bought from commercial sources.

Fusion protein constructs: Plasmids encoding RLuc8 and YPet-β-arrestin2 were provided by Dr. Ralf Jockers (Institut Cochin, Paris, France) and human cDNAs for the glucagon family receptors were amplified from human cDNA using standard RT-PCR. Fusion proteins consisting of full length receptors c-terminally fused in frame to RLuc8 or YPet were generated by PCR and DNA recombination using Invitrogen's Multisite Gateway® Pro technology (Invitrogen Corporation, Carlsbad, CA, USA). By standard PCR, receptors, YPet and RLuc8 were amplified using primers that added specific Gateway® recombination sites for two-way fusion to the products according to the manufacturer's recommendations. The N-terminal fusion protein elements (receptors) were inserted without stop codon into pDONR 1-5r vectors and the C-terminal elements RLuc8 or YPet into pDONR 5-2. Receptor fusion proteins were finally generated by site-specific DNA recombination using one receptor-containing pDONR plasmid, one RLuc8- or YPet-containing pDONR plasmid, and one expression plasmid containing an acceptor Gateway® cassette.

Tissue culture: Human Embryonic Kidney cells (HEK 293T) were cultured in DMEM containing 100 Units/ml penicillin, 100 µg/ml streptomycin (all Invitrogen) and 10% FCS (Chemie Brunschwig, Basel, Switzerland) in a humidified incubator at 37°C/ 5% CO_2. Cells were transiently transfected in 12- or 6-well plates (Nunc GmbH

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

& Co. KG, Langenselbold, Germany) at 50-60% confluence using 10 µl or 20 µl Optifect™ reagent (Invitrogen) and 2 µg or 4 µg DNA, according to the manufacturer's protocol. The cells were used 48 h after transfection.

One day prior to the BRET or calcium experiments, cells were detached with trypsin/EDTA (Invitrogen) and seeded in black clear-bottom 384-well plates (Nunc) coated with 10µg/ml poly-L-ornithin (Sigma-Aldrich, St. Louis, MO, USA), at 20-30'000 cells/well in growth medium.

BRET measurements: On the day of the experiment, the medium was aspirated, cells were serum-starved in HBSS++ (Invitrogen) for 60 min at 37°C, washed with PBS (Invitrogen), and the bioluminescent reaction was started by addition of coelenterazine H (Dalton Pharma Services, Toronto, Canada) at a final concentration of 6 µM in a 45 µl HBSS++/well. 5 min after the addition of Coelenterazine H the dynamic BRET response was read on a FLIPR® Tetra high throughput screening system (Molecular Devices, Sunnyvale, CA, USA). Light emission of the donor RLuc8 (emission peak: 487 nm) was detected with a bandpass emission filter of 460 +/-20 nm and the acceptor YPet (emission peak: 535 nm) was detected at 556 +/-30 nm. Both signals were recorded using equal exposure times and maximum gain settings with read intervals of 3-6 sec, depending on signal strength. All 384 wells were recorded simultaneously, allowing for a dynamic readout. Ligands were added online by transferring 15 µl from a 4x concentrated addition plate with a multi-tip head to the read plate. In two addition protocols, the second ligand was added by transferring an additional 20 µl from a second 4x concentrated addition plate during the read. The signal was read for 10 to 60 min and the BRET ratio was defined as [(556 +/-30 nm)/(460 +/-20 nm)-*cf*], where the correction factor *cf* corresponds to [(556 +/-30 nm)/(460 +/-20 nm)] of a receptor-RLuc8 BRET donor expressed in the

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

absence of the YPet acceptor protein. Dose response curves were graphed by plotting the average net BRET over 5 to 10 min after a single addition of increasing concentrations of ligand.

Calcium measurements: On the day of the experiment, cells were washed 3x with PBS and incubated with 40 µl per well calcium buffer (143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 0.1% glucose, 0.125 mM sulfinpyrazone, 1 mM $CaCl_2$) containing 3 µM of the green fluorescent calcium indicator fluo-4 AM (Invitrogen) for one hour at 37°C/ 5% CO_2. Cells were washed 3x with PBS and incubated with 45 µl of calcium buffer for another 3.5 h at room temperature in the dark. The calcium response was read on the FLIPR® Tetra using LED excitation (470-495 nm) in combination with a band pass emission filter (545 +/- 30 nm) to measure the calcium-induced fluo4-AM fluorescence. Ligands were added online by transferring 15 µl from a 4x concentrated addition plate with a multi-tip head to the read plate. Dose response curves were calculated by subtracting the minimal from the maximal value of the dynamic calcium-response over 5 min (max-min). Each data point was measured in duplicates.

Fluorescent imaging: Transfected cells were seeded on 10 µg/ml poly-L-ornithin (Sigma-Aldrich) coated coverslips 24 h after transfection, washed after 48 h, incubated with ligand in HBSS++ for 30 min at room temperature, fixed with PBS (4% PFA) and mounted with Mowiol (Sigma-Aldrich) on microscope slides. Images were obtained with an Axiovert 200 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) using YFP and ECFP filter sets and a 63x oil immersion objective. Images were digitally treated using ImageJ (http://rsbweb.nih.gov/ij/).

Data analysis: The dose response curves were fitted to a four-parameter nonlinear regression/sigmoidal dose-response equation using Prism 5.0® (Graph Pad Software

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Inc, San Diego, USA) allowing determination of EC_{50} values. Each curve data point was measured as duplicate data points if not indicated otherwise. Statistical relevance when comparing the effects of ligands was determined using one-way ANOVA followed by Tukey multiple comparison test when comparing more than two conditions, or by unpaired two-tailed t-test when comparing two conditions using Prism 5.0®

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The relationship between the glucagon-family ligands glucagon, GLP-1, GLP-2, and GIP was compared by aligning primary protein structure (Figure 1). Crystal structures for the complexes of GLP-1 and GIP with the extracellular domains of their respective receptors have been solved recently (Underwood et al., 2010;Parthier et al., 2007) and residues that could be modeled by the authors are underlined. The four peptides share a glucagon-like core structure of 27 amino acids (AAs) with highly conserved N-terminal (AAs 1-9 of glucagon) and C-terminal domains (AAs 20-28).

A marked difference between the ligands is found in the last C-terminal residues of each peptide that are non-conserved and variable in size, with glucagon possessing the shortest and GIP the longest C-tail. The precise interactions of those tails with the receptor are unknown. Glucagon is evolutionary more closely related to GIP than to GLP-1 or -2 despite that fact that GIP is derived from a separate precursor protein.

To assess possible ligand-induced interactions between the receptors of the glucagon-family, HEK cells were co-transfected with equal amounts of plasmid encoding receptors fused to RLuc8 or YPet, respectively. Heteromer combinations of receptors were tested in two configurations, swapping the RLuc8 and YPet tags for each receptor combination, to detect interactions independent of the tagging. The subcellular localization of the receptors was assessed by immunofluorescence (anti-RLuc) or direct fluorescence microscopy (YPet) 48 h after transfection (data not shown). The receptors were mainly located at the cell membrane in all combinations tested except for the combination GIPR-RLuc8/GIPR-YPet where aggregation in the ER/golgi was observed. The basal BRET ratio between the receptor constructs was determined and changes in the energy transfer after addition of ligand were calculated by subtracting the BRET signal obtained after ligand addition from the basal ratio. In

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

the following sections, if not indicated differently, "GLP-1" will refer to the physiological agonist GLP-1(7-36)NH_2.

Homomeric interactions: In HEK cells co-expressing the same receptor coupled to RLuc8 or YPet, basal BRET signals were observed for GIPR/GIPR and GlucR/GlucR, and to a lesser extent for GLP-2/GLP-2 and GLP-1/GLP-1 (Figure 2, bottom). Addition of GIP or glucagon induced a small reduction of the energy transfer between the GIPR/GIPR and GlucR/GlucR, respectively (Figure 2, top).

Heteromeric interactions: Heteromer formation was assessed by double-transfecting cells with different BRET-compatible receptor combinations. Each combination was tested once as "receptorA-RLuc8/receptorB-YPet" and once with the same receptors and switched tags fused (Figure 2). **GLP-1R/GIPR:** A strong, highly significant increase of the BRET ratio was observed upon GLP-1 stimulation of cells expressing GLP-1R-RLuc8/GIPR-YPet ($p<0.001$) suggesting the recruitment of a heteromer or a conformational change in an existing one. The same effect was observed in cells expressing GLP-1R-YPet/GIPR-RLuc8 ($p<0.01$), confirming independence of BRET-tag orientation. GIP induced a small decrease of the basal BRET level, suggesting inverse effects of the ligands. We estimated the GLP-1R expression level in the BRET experiments by fluorescent ligand binding and compared it to the insulinoma cell line INS-1E, a physiological reference (Supplementary Figure 1), and found an average 17.2-fold overexpression. Lowering the BRET receptor expression levels to an estimated 2.4-fold and 1.1-fold compared to INS-1E, we still observed GLP-1 induced receptor heteromerization (Supplementary Figure 2) but no GIP effect on the basal BRET. The detection limit of the luminescence reader used in this study was reached at low receptor expression levels. Such conditions could therefore not be used for the solid generation of data. **GLP-1R/GlucR:** A small but significant decrease of the

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

basal BRET ratio was induced by stimulation with GLP-1 ($p<0.001$), but not with glucagon in cells expressing GLP-1R-RLuc8 and GlucR-YPet. **GlucR/GLP-2R:** A decrease of the basal BRET ratio of GlucR-Luc/GLP-2R-FP was induced after stimulation with glucagon ($p<0.05$) or GLP-2 ($p<0.01$). The same tendency could be observed when the BRET tags were inverted, however the response was less pronounced. In all other receptor combinations, ligand addition did not significantly affect the basal BRET ratio. As ligand-induced increase in BRET was only seen with GLP-1R/GIPR, this unique interaction was further characterized. **BRET donor saturation experiments:** Next, the nature of the interactions between GLP-1R and GIPR was determined in BRET donor saturation experiments (Issad and Jockers, 2006). Cells were co-transfected with a fixed amount of the BRET donor plasmid and increasing amounts of the acceptor plasmid. The energy transfer between the receptors was quantified as net BRET in the presence or absence of saturating concentrations of ligand. An increase of BRET between GIPR and GLP-1R was observed with increasing concentrations of transfected GIPR-YPet plasmid in buffer conditions (Figure 3A), but no saturation of the curve was observed. Presence of GLP-1 increased the BRET in three independent experiments leading to a saturation of the curve that reached maximal BRET interaction level at $BRET_{max}$= 0.66 +/- 0.05 S.E.M. and to a left-shift of the curve, allowing the calculation of a half-saturation ratio $BRET_{50}$= 1.27 +/- 0.20 S.E.M. where 50% of donor are occupied by the acceptor (Issad and Jockers, 2006). In a reverse BRET acceptor saturation experiment, decreasing concentrations of GLP-1R-Rluc8 produced increasing BRET signals against a fixed concentration of GIPR-YPet in the presence of GLP-1, confirming the specificity of this interaction, also at low levels of GLP-1R (Supplementary Figure 3). The saturation curves of GIPR alone or GLP-1R alone showed a ligand induced

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

reduction of BRET when the donor was present in excess, suggesting that these receptors interact as homomers and that a reduction of affinity or a conformational change occurs upon ligand binding (Figure 3C,D). In the control experiments a quasi-linear, non-saturated BRET curve was observed suggesting non-specific, random interactions between the non-glucagon receptor M4R-RLuc8 (class A) and GLP-1R-YPet. The presence of GLP-1 did not alter the signal, showing that the GLP-1 effect of heteromerization was receptor specific (Figure 3B).

GLP-1 and GIP have opposing effects on receptor heteromerization in the BRET system. The pharmacology of the heteromerization of GLP-1R and GIPR was characterized by transfecting HEK cells with equal amounts of GLP-1R-RLuc8 and GIPR-YPet plasmids. The dynamics of the GLP-1 response in BRET was monitored by online addition of increasing concentrations of the ligand, which resulted in rapid formation of the BRET response (Figure 4A). Opposing effects and the dynamics of GLP-1 and GIP on the receptor recruitment in BRET were tested by subsequent addition of both ligands (Figure 4B). GLP-1 addition induced a recruitment of GLP-1R to GIPR that resulted in a stable maximal BRET signal obtained after 0.5 min. Addition of GIP 1 or 5 min after the addition of GLP-1 reversed the GLP-1-induced BRET augmentation to baseline levels, which was completed 3-4 min after GIP addition. GIP was still effective in decreasing the BRET signal 5 min after GLP-1 addition, indicating that the receptor heteromer was still present at the ligand-accessible cell surface.

GLP-1, GLP-1(9-36) and GLP-2 induce receptor heteromerization. GLP-1R and GIPR antagonists GLP-1(9-36) and (Pro^3)GIP were characterized in full dose. Both ligands are known to bind to their receptors, but their receptor-activating N-terminal residues are truncated or mutated. Their BRET response was compared to the GLP-1

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

and GIP response (Figure 5A). GLP-1 and GLP-1(9-36) were equally potent in recruiting the receptor heteromer ($EC_{(50)}$= 243 nM +/- 95 nM and 266 nM +/- 145 nM S.E.M.) but GLP-1(9-36) surpassed the maximal response achieved with GLP-1. GIP caused a decrease of the basal BRET ratio ($IC_{(50)}$= 33 nM +/- 2.1 nM) suggesting some pre-existing interaction between the receptors in the absence of ligand. Likewise, GIPR antagonist (Pro^3)GIP had a similar effect as GIP on the heteromer; both antagonized $EC_{(80)}$ concentrations (1.3 μM) of GLP-1 ($IC_{(50)}$= 135 nM +/- 43 nM and 369 nM +/- 188 nM S.E.M.) and decreased the BRET signal below the buffer baseline, similar to effects of GIP alone.

To further characterize the pharmacology of the GIP-inhibition of receptor heteromerization, dose responses of GLP-1 were carried out at increasing concentrations of GIP, which resulted in a decrease of the initial BRET value and a right-shift of the GLP-1 dose response curve (Figure 5B). High doses of GLP-1 could overcome the GIP inhibition to obtain the same maximal response as GLP-1 alone. For the heteromerization, this pharmacological profile suggests an orthosteric competition of GLP-1 and GIP for the same binding site on the receptor.

Further naturally occurring ligands, and derived agonists and antagonists of GLP-1R and GIPR were tested for their potential to induce a heteromerization between the receptors (Figure 5C). GLP-1, GLP-1(9-36), and GLP-2 ($EC_{(50)}$= 407 nM +/- 68 nM) significantly increased the BRET signal between the receptors ($p<0.001$) while glucagon, Oxyntomodulin, Exendin-4, known activators of GLP-1R, did not induce heteromerization. On the contrary, Exendin-4 decreased the basal BRET interaction in a similar way to GIP and (Pro^3)GIP, whereas the functional antagonist Exendin (9-39) showed no activity. Probing those ligands against an $EC_{(80)}$ concentration of GLP-1

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

confirmed an effective disruption of the BRET complex by Exendin-4 ($p<0.05$), GIP and (Pro^3)GIP ($p<0.001$).

Receptor internalization. As similar effects on receptor interaction were observed with agonist and antagonists in BRET, receptor localization was studied to monitor internalization of each receptor and to detect possible GLP-1R/GIPR complexes. Cells were transfected with fluorescently tagged GLP-1R (ECFP) and GIPR (YPet). In the non-treated condition, both receptors were located at the membrane (Figure 6A,E). GLP-1 (6B,F), Exendin-4 (6D,H) and glucagon (6E,I) caused internalization of mainly GLP-1R while GIPR remained at the membrane. In contrast, GLP-1(9-36) (6C,G) and GLP-2 (6I,M) did not have an effect on receptor internalization. Similarly, GIP (6K,O) internalized mainly the GIPR while (Pro^3)GIP (6L,P) had no effect on either receptor. Recruitment and disruption of the GLP-1R/GIPR heteromer by GLP-1(9-36), GLP-2, and (Pro^3)GIP (Figure 5) seems to represent a mechanism that was prior to and independent of receptor internalization.

Co-expression of GLP-1R and GIPR alters the GLP-1 induced, but not the GIP-induced calcium response in HEK cells. GLP-1R and GIPR both induce a cellular calcium flux in response to ligand, raising the question if allosteric regulation in a heteromeric complex of those receptors would result in altered calcium pharmacology. HEK cells transfected with GLP-1R alone or GIPR alone induced calcium flux in response to their respective ligands, with no effect of the other ligand at concentrations < 3 μM (Figure 7A). Co-expression of GLP-1R with GLP-2R and GlucR had no effect on the GLP-1 response (Figure 7B). Co-expression of GLP-1R and GIPR, however resulted in an altered, flattened pharmacological response to GLP-1, with a significant change of the $EC_{(50)}$ ($p<0.05$) and a highly significant effect on the Hill slope ($p<0.001$) compared to cells expressing GLP-1R alone (Table 1).

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Under these conditions, lower doses of GLP-1 (0.3 nM) stimulated calcium flux and the dose response curve approached the pharmacological profile of the GIP response. The presence of GLP-1R did not affect the profile of the GIP response compared to cells expressing the GIPR alone. This observation was in line with the finding that GLP-1 but not GIP induced a heteromerization of the two receptors in BRET and suggests a pharmacological role of the receptor heteromerization in calcium signalling.

Presence of GIPR influences GLP-1-induced β-arrestin recruitment to GLP-1R. In pancreatic cells, β-arrestin recruitment contributes importantly to GLP-1 induced insulin release (Dalle et al., 2011). Based on BRET results that showed a recruitment of β-arrestin2 to GLP-1R (Jorgensen et al., 2007) we used a similar approach to evaluate effects of the heteromerization on the arrestin recruitment.

Cells transfected with luciferase-tagged GLP-1R and YPet-β-arrestin2 responded to GLP-1 with a dose-dependent increase of the BRET signal (Figure 8A). Co-expression of GIPR markedly decreased the BRET response of GLP-1 at concentrations >30 nM (Figure 8B), reducing the maximal plateau of arrestin recruitment to GLP-1R by ~25% at potencies of GLP-1 that remained comparable ($EC_{(50)}$ GLP1-R = 32.1 nM; $EC_{(50)}$ GLP1-R/GIPR = 17.7 nM). As the GLP-1 effect on receptor heteromerization was observed at similar concentrations (Figure 5A) we assessed the possibility of reversing this effect on arrestin-recruitment by addition of GIP. In presence of a fixed concentration of GIP (100 nM), the maximal response of GLP-1 on the co-expressed receptors was similar to the level of the GLP-1 response on GLP-1R alone (Figure 8A,B). Additionally, at saturating concentrations of GLP-1 (1 μM), GIP was able to further increase the BRET signal in a dose-dependent manner. The addition of 100 nM GIP alone did not induce a BRET signal in both

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

transfection conditions, with or without the GIPR present. Likewise, in the absence of the GIPR, co-addition of a fixed concentration of GIP (100nM) did not have an effect on the GLP-1 response and increasing concentrations of GIP did not alter the maximal GLP-1 response, suggesting no activity of GIP on GLP-1R.

The arrestin recruitment results create a link to the receptor heteromerization phenomenon observed, suggesting a physiologically relevant mechanism of allosteric regulation through this interaction and allow for the development of a heteromerization model.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In the present study, we grouped functionally related class B receptors, known to be co-expressed in the same cells that respond to the closely related ligands glucagon, GLP-1, GLP-2, and GIP that are involved in the glucose homeostasis (Baggio and Drucker, 2007). We observed, for the first time, a dose-dependent GLP-1 induced formation of a heteromer between GLP-1R and GIPR. This effect was still detectable when receptor expression levels were lowered to match those measured in a pancreatic cell line endogenously expressing GLP-1R (Supplementary Figure 2), excluding an artefact due to receptor overexpression. The functional GLP-1R antagonist GLP-1(9-36) and GLP-2 mimicked the GLP-1 effect. No such behavior was observed between other members of the glucagon receptor family. On the contrary, both GIP and (Pro^3)GIP dose-dependently inhibited the GLP-1-induced GLP-1R/GIPR heteromerization. BRET donor saturation experiments indicate that a specific receptor recruitment rather than only a conformational change of an already existing heteromer is induced by GLP-1 (Issad and Jockers, 2006). This is, to our knowledge, the first example of ligand-induced receptor heteromerization in class B.

In a study that tested the potential of class B prototypic SecR to form intra-family heteromers with nine other class B GPCRs (including GLP-1R and GLP-2R) in response to ligand, the authors observed constitutive interactions of SecR with most of the receptors tested and ligand-induced decrease of heteromerization (Harikumar et al., 2008). Interestingly, no complex formation upon ligand binding was reported for the receptors tested. We also observed reduction of the basal BRET between GLP-1R and GIPR after GIP addition (Figure 5), and a small but significant ligand-induced BRET decrease between GlucR/GLP-1R and GlucR/GLP-2R. Confirmation of such small changes in the BRET ratio at lower expression levels is technically challenging

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

using our setup. Therefore, we cannot exclude that the basal receptor interaction observed comes from random collisions of overexpressed receptors. Consequently, ligand-induced BRET decreases could be the result of receptor internalization.

Class B ligands are considered as “two-domain” peptides. Their C-terminal domain conveys receptor specificity by binding to the ECD, while the N-terminal domain activates the receptor via the transmembrane domain (Hoare, 2005). GLP-1 and its antagonists, GLP-1(9-36) and GLP-2, induced receptor heteromerization in the BRET assay, but GLP-1(9-36) and GLP-2 did not alter the surface localization of the receptors (Figure 6C,G,J,N), which suggests that this receptor heteromerization is independent of GLP-1R activation, making an involvement of the C-terminal receptor binding domain of the ligand more likely. In contrast, GLP-1 induced internalization of GLP-1R while most of GIPR remained at the membrane (Figure 6B,F). This indicates that either most of the GLP-1R population is activated and internalized independent of the GIPR or that the heteromeric complex is dissolved before the GLP-1R is internalized. The finding that GLP-1(9-36) induced a stronger BRET response than GLP-1 supports this possibility (Figure 5C). The BRET heteromerization of GLP-1R/GIPR reached its maximum 10-30 sec after ligand addition (Figure 4A) whereas GLP1-R internalization could be observed 1-2 min after addition, indicating two independent sequential events. The possibility to disrupt the BRET complex induced by GLP-1 with a subsequent addition of GIP even after 5 min (Figure 4B) shows that the heteromers stay at the cell surface or that they are internalized and cycled back to the membrane where GIP can bind.

Recruitment of the GLP-1R/GIP heteromer by BRET was detectable at ligand concentrations > 10 nM, higher than the 5-30 pM GLP-1 found in human blood (Vilsboll et al., 2001). In the lamina propria of the gastrointestinal mucosa where

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

GLP-1 is released from L-cells, locally high concentrations of GLP-1 could act in a paracrine way, for example, on afferent nerve terminals that co-express GLP-1R and, however to much lower extent, GIPR (Nakagawa et al., 2004). GLP-1 contributes to insulin release and glucose increase via afferent sensory neurons (Ahren, 2004). Neuronal GIPR expression has also been reported for various cell types of the central and peripheral nervous system and was linked to endo- or paracrine signaling (Buhren et al., 2009). Half of the GLP-1 produced in the intestine is metabolized to GLP-1(9-36) as it enters the circulation (Hansen et al., 1999) making this antagonist the predominant bio-available form of GLP-1. Our observation that GLP-1(9-36) can induce receptor heteromerization adds to the debate about this entity's physiological role.

Unlike GLP-1, the lizard-derived bioactive analogue Exendin-4 did not induce heteromerization. Exendin-4 (39 AA) possesses an extended C-terminus similar to that of GIP (42AA). These residues have not been resolved in ligand/receptor crystal structures (Runge et al., 2008;Underwood et al., 2010;Parthier et al., 2007) and Exendin-4 may interact with regions of the receptor ECD important for receptor heteromerization differently than the relatively short heteromer-inducing ligands GLP-1, GLP-1(9-36) and GLP-2 (Figure 1). A short consensus repeat region situated near the C-terminus of the bound ligand, known to promote protein-protein interactions, is present in the ECD of the CRF-R2 receptor and is conserved in the GLP-1R (Grace et al., 2004) provoking the question of a possible involvement in GLP-1R/GIPR heteromerization.

GIP and (Pro^3)GIP both inhibited GLP-1 induced receptor heteromerization raising the question if those ligands act by binding to a separate binding site on the heteromer or by orthosterically competing with GLP-1 for the same binding site. GIP did not

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

activate GLP-1R expressed alone in the calcium response and in arrestin-recruitment. High concentrations of GIP did not affect the pharmacological response of GLP-1R to GLP-1, suggesting that GIP does not compete with GLP-1 for the ligand binding site on the GLP-1R (Figure 8A). The BRET heteromerization shift experiment (Figure 5B), however, suggests a competition mechanism as the BRET-decreasing GIP effect can always be overcome by high concentrations of GLP-1, implying a competition of GLP-1 and GIP for the same binding site on the GIPR.

In the arrestin recruitment and calcium response experiments, an altered GLP-1 pharmacology of the GLP-1R with reduced amplitude was observed in presence of the GIPR at concentrations of GLP-1 >30 nM. This corresponds to the concentrations at which receptor heteromerization became apparent (Figure 5A), whereas GLP-1R calcium responses and arrestin-recruitment through GLP-1 were already detectable at lower concentrations. This raises the possibility that the activation of GLP-1R is induced by a high affinity binding of GLP-1 to its receptor, whereas GLP-1R/GIPR heteromerization is mediated by a low-affinity binding of GLP-1 to the GIPR.

Indeed, when GLP-1R and GIPR were co-expressed in the arrestin experiment, co-incubation with GIP rescued the normal GLP-1 pharmacology, restoring the identical response of GLP-1R when expressed alone (Figure 8B). This suggests that the changed pharmacological profile results from allosteric regulation of GLP-1R through the recruitment of GIPR at high GLP-1 concentrations.

Based on the results, a model for GLP-1R/GIPR heteromerization can be derived (Figure 9). In this model, GLP-1 serves as a high affinity ligand for GLP-1R, inducing functional responses at low concentrations (Figure 9A). At higher concentrations and in absence of GIP, GLP-1 becomes also a low affinity ligand for GIPR, and its binding induces a receptor heteromer in which both GLP-1R and GIPR bind GLP-1

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

(B). As the maximal response in our calcium and β-arrestin recruitment experiments resulted in a decrease of the maximal response, the interaction with the GIPR seems to represent a form of allosteric regulation of the GLP-1R. Finally, addition of the high affinity ligand GIP can displace GLP-1 from the GIPR, thereby dissolving the receptor heteromer and restoring the normal GLP-1R pharmacology (Figure 9C).

Oligomerization of receptors can have multiple effects on the behavior of each receptor in the complex. Ligand binding can increase the affinity of the receptor, modulate the signaling response, affect the potencies of agonists, or induce a switching of G-protein coupling (Smith and Milligan, 2010). GLP-1R can also couple to different G-proteins (Montrose-Rafizadeh et al., 1999) and in our study we showed that co-expression of GLP-1R and GIPR changed the $EC_{(50)}$ and the Hill slope of the GLP-1 response compared to GLP-1R expressed alone (Figure7). Increased calcium flux at low concentrations and a reduced maximal response may reflect an altered G-protein coupling behavior, for example of Gq, to the heteromeric GLP-1R/GIPR complex, giving the GLP-1 response a more GIP-like character. Changes in the Hill slope have been described as characteristic pharmacological “fingerprints” for GPCR dimers and their allosteric behavior (Franco et al., 2008). We also observed a decreased maximal BRET response of the arrestin recruitment to GLP-1R when co-expressed with GIPR (Figure 8). If this decrease of BRET reflects a reduction of the total number of GLP-1R/β-arrestin complexes formed in when GIPR is present, the heteromerization with GIPR may potentially present a form of protection from receptor desensitization.

In conclusion the present study shows, to our knowledge for the first time, that class B GPCRs can form ligand-induced heteromers in a heterologous overexpression system. GIPR and GLP-1R are co-expressed in pancreatic and nerve cells, and their role in

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Type 2 diabetes makes them interesting targets for drug development. Further evidence is needed to prove the in vivo existence and pharmacological relevance of such heteromers and understanding their role may open the door to possible pinpointed therapies targeting the GLP-1R/GIP heteromer.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

We would like to thank Dr. Ralf Jockers (Institut Cochin, Paris, France) who kindly provided the plasmids encoding RLuc8 and YPet-β-arrestin2 for research purposes and who assisted with the interpretation of BRET results.

Authorship Contributions

Participated in research design:

Schelshorn, Lütjens, Mutel V, Breton, Hampe.

Conducted experiments:

Joly, Schelshorn, Mutel S.

Cloned receptor fusion proteins: Mutel S, Hampe, Schelshorn

Performed data analysis: Schelshorn, Joly, Mutel S.

Wrote or contributed to the writing of the manuscript: Schelshorn, Joly, Lütjens, Breton, Mutel, Hampe

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Reference List

Ahren B (2004) Sensory Nerves Contribute to Insulin Secretion by Glucagon-Like Peptide-1 in Mice. *Am J Physiol Regul Integr Comp Physiol* **286**:R269-R272.

Alvarez E, Martinez M D, Roncero I, Chowen J A, Garcia-Cuartero B, Gispert J D, Sanz C, Vazquez P, Maldonado A, de C J, Desco M, Pozo M A and Blazquez E (2005) The Expression of GLP-1 Receptor MRNA and Protein Allows the Effect of GLP-1 on Glucose Metabolism in the Human Hypothalamus and Brainstem. *J Neurochem* **92**:798-806.

Ayoub MA, Couturier C, Lucas-Meunier E, Angers S, Fossier P, Bouvier M and Jockers R (2002) Monitoring of Ligand-Independent Dimerization and Ligand-Induced Conformational Changes of Melatonin Receptors in Living Cells by Bioluminescence Resonance Energy Transfer. *J Biol Chem* **277**:21522-21528.

Baggio LL and Drucker D J (2007) Biology of Incretins: GLP-1 and GIP. *Gastroenterology* **132**:2131-2157.

Bockaert J, Fagni L, Dumuis A and Marin P (2004) GPCR Interacting Proteins (GIP). *Pharmacol Ther* **103**:203-221.

Bouvier M (2001) Oligomerization of G-Protein-Coupled Transmitter Receptors. *Nat Rev Neurosci* **2**:274-286.

Brubaker PL and Drucker D J (2002) Structure-Function of the Glucagon Receptor Family of G Protein-Coupled Receptors: the Glucagon, GIP, GLP-1, and GLP-2 Receptors. *Receptors Channels* **8**:179-188.

Buhren BA, Gasis M, Thorens B, Muller H W and Bosse F (2009) Glucose-Dependent Insulinotropic Polypeptide (GIP) and Its Receptor (GIPR): Cellular Localization, Lesion-Affected Expression, and Impaired Regenerative Axonal Growth. *J Neurosci Res* **87**:1858-1870.

Cabello N, Gandia J, Bertarelli D C, Watanabe M, Lluis C, Franco R, Ferre S, Lujan R and Ciruela F (2009) Metabotropic Glutamate Type 5, Dopamine D_2 and Adenosine A_{2a} Receptors Form Higher-Order Oligomers in Living Cells. *J Neurochem* **109**:1497-1507.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson T J, Higgins D G and Thompson J D (2003) Multiple Sequence Alignment With the Clustal Series of Programs. *Nucleic Acids Res* **31**:3497-3500.

Dalle S, Ravier M A and Bertrand G (2011) Emerging Roles for B-Arrestin-1 in the Control of the Pancreatic B-Cell Function and Mass: New Therapeutic Strategies and Consequences for Drug Screening. *Cell Signalling* **23**:522-528.

Doumazane E, Scholler P, Zwier J M, Eric T, Rondard P and Pin J P (2011) A New Approach to Analyze Cell Surface Protein Complexes Reveals Specific Heterodimeric Metabotropic Glutamate Receptors. *FASEB J* **25**:66-77.

Franco R, Casado V, Cortes A, Mallol J, Ciruela F, Ferre S, Lluis C and Canela E I (2008) G-Protein-Coupled Receptor Heteromers: Function and Ligand Pharmacology. *Br J Pharmacol* **153 Suppl 1**:S90-S98.

Grace CR, Perrin M H, Digruccio M R, Miller C L, Rivier J E, Vale W W and Riek R (2004) NMR Structure and Peptide Hormone Binding Site of the First Extracellular Domain of a Type B1 G Protein-Coupled Receptor. *Proc Natl Acad Sci USA* **101**:12836-12841.

Han Y, Moreira I S, Urizar E, Weinstein H and Javitch J A (2009) Allosteric Communication Between Protomers of Dopamine Class A GPCR Dimers Modulates Activation. *Nat Chem Biol* **5**:688-695.

Hansen L, Deacon C F, Orskov C and Holst J J (1999) Glucagon-Like Peptide-1-(7-36)Amide Is Transformed to Glucagon-Like Peptide-1-(9-36)Amide by Dipeptidyl Peptidase IV in the Capillaries Supplying the L Cells of the Porcine Intestine. *Endocrinology* **140**:5356-5363.

Harikumar KG, Morfis M M, Lisenbee C S, Sexton P M and Miller L J (2006) Constitutive Formation of Oligomeric Complexes Between Family B G Protein-Coupled Vasoactive Intestinal Polypeptide and Secretin Receptors. *Mol Pharmacol* **69**:363-373.

Harikumar KG, Morfis M M, Sexton P M and Miller L J (2008) Pattern of Intra-Family Hetero-Oligomerization Involving the G-Protein-Coupled Secretin Receptor. *J Mol Neurosci* **36**:279-285.

Hoare SR (2005) Mechanisms of Peptide and Nonpeptide Ligand Binding to Class B G-Protein-Coupled Receptors. *Drug Discov Today* **10**:417-427.

Issad T and Jockers R (2006) Bioluminescence Resonance Energy Transfer (BRET) to Monitor Protein-Protein Interactions. *Methods Mol Biol* **332**:195-209.

Jorgensen R, Kubale V, Vrecl M, Schwartz T W and Elling C E (2007) Oxyntomodulin Differentially Affects Glucagon-Like Peptide-1 Receptor Beta-Arrestin Recruitment and Signaling Through Galpha(s). *J Pharmacol Exp Ther* **322**:148-154.

Kamal M, Marquez M, Vauthier V, Leloire A, Froguel P, Jockers R and Couturier C (2009) Improved Donor/Acceptor BRET Couples for Monitoring B-Arrestin Recruitment to G Protein-Coupled Receptors. *Biotechnol J* **4**:1337-1344.

Li X, Staszewski L, Xu H, Durick K, Zoller M and Adler E (2002) Human Receptors for Sweet and Umami Taste. *Proc Natl Acad Sci USA* **99**:4692-4696.

Marcellino D, Ferre S, Casado V, Cortes A, Le F B, Mazzola C, Drago F, Saur O, Stark H, Soriano A, Barnes C, Goldberg S R, Lluis C, Fuxe K and Franco R (2008) Identification of Dopamine D_1-D_3 Receptor Heteromers. Indications for a Role of Synergistic D_1-D_3 Receptor Interactions in the Striatum. *J Biol Chem* **283**:26016-26025.

Matsushita S, Nakata H, Kubo Y and Tateyama M (2010) Ligand-Induced Rearrangements of the GABAb Receptor Revealed by Fluorescence Resonance Energy Transfer(FRET). *J Biol Chem*.

Mayo KE, Miller L J, Bataille D, Dalle S, Goke B, Thorens B and Drucker D J (2003) International Union of Pharmacology. XXXV. The Glucagon Receptor Family. *Pharmacol Rev* **55**:167-194.

Montrose-Rafizadeh C, Avdonin P, Garant M J, Rodgers B D, Kole S, Yang H, Levine M A, Schwindinger W and Bernier M (1999) Pancreatic Glucagon-Like Peptide-1 Receptor Couples to Multiple G Proteins and Activates Mitogen-Activated Protein Kinase Pathways in Chinese Hamster Ovary Cells. *Endocrinology* **140**:1132-1140.

Nakagawa A, Satake H, Nakabayashi H, Nishizawa M, Furuya K, Nakano S, Kigoshi T, Nakayama K and Uchida K (2004) Receptor Gene Expression of Glucagon-Like Peptide-1, but Not Glucose-Dependent Insulinotropic Polypeptide, in Rat Nodose Ganglion Cells. *Auton Neurosci* **110**:36-43.

Parthier C, Kleinschmidt M, Neumann P, Rudolph R, Manhart S, Schlenzig D, Fanghanel J, Rahfeld J U, Demuth H U and Stubbs M T (2007) Crystal Structure of the Incretin-Bound Extracellular Domain of a G Protein-Coupled Receptor. *Proc Natl Acad Sci USA* **104**:13942-13947.

Patel RC, Kumar U, Lamb D C, Eid J S, Rocheville M, Grant M, Rani A, Hazlett T, Patel S C, Gratton E and Patel Y C (2002) Ligand Binding to Somatostatin Receptors Induces Receptor-Specific Oligomer Formation in Live Cells. *Proc Natl Acad Sci USA* **99**:3294-3299.

Pfleger KD and Eidne K A (2005) Monitoring the Formation of Dynamic G-Protein-Coupled Receptor-Protein Complexes in Living Cells. *Biochem J* **385**:625-637.

Pinard A, Seddik R and Bettler B (2010) GABAB Receptors: Physiological Functions and Mechanisms of Diversity. *Adv Pharmacol* **58**:231-255.

Rashid AJ, So C H, Kong M M, Furtak T, El-Ghundi M, Cheng R, O'Dowd B F and George S R (2007) D1-D2 Dopamine Receptor Heterooligomers With Unique Pharmacology Are Coupled to Rapid Activation of G_q/11 in the Striatum. *Proc Natl Acad Sci USA* **104**:654-659.

Rocheville M, Lange D C, Kumar U, Patel S C, Patel R C and Patel Y C (2000) Receptors for Dopamine and Somatostatin: Formation of Hetero-Oligomers With Enhanced Functional Activity. *Science* **288**:154-157.

Runge S, Thogersen H, Madsen K, Lau J and Rudolph R (2008) Crystal Structure of the Ligand-Bound Glucagon-Like Peptide-1 Receptor Extracellular Domain. *J Biol Chem* **283**:11340-11347.

Smith NJ and Milligan G (2010) Allostery at G Protein-Coupled Receptor Homo- and Heteromers: Uncharted Pharmacological Landscapes. *Pharmacol Rev* **62**:701-725.

Underwood CR, Garibay P, Knudsen L B, Hastrup S, Peters G H, Rudolph R and Reedtz-Runge S (2010) Crystal Structure of Glucagon-Like Peptide-1 in Complex With the Extracellular Domain of the Glucagon-Like Peptide-1 Receptor. *J Biol Chem* **285**:723-730.

Vilsboll T, Krarup T, Deacon C F, Madsbad S and Holst J J (2001) Reduced Postprandial Concentrations of Intact Biologically Active Glucagon-Like Peptide 1 in Type 2 Diabetic Patients. *Diabetes* **50**:609-613.

Young SF, Griffante C and Aguilera G (2007) Dimerization Between Vasopressin V1b and Corticotropin Releasing Hormone Type 1 Receptors. *Cell Mol Neurobiol* **27**:439-461.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Figure 1: Protein sequence alignment of human glucagon, GLP-1, GLP-2, and GIP. The amino acid alignment of the ligands was constructed with ClustalW (Chenna et al., 2003). Residues of GLP-1 and GIP that were resolved by crystallisation with the extracellular domains of GLP-1R and GIPR are underlined.

"*" = fully conserved (identical residues); ":" = highly conserved (conserved substitutions of same polarity or hydrophobic); "." = semi-conserved (substitutions of similar shape)

Figure 2: Interactions between the glucagon family receptors by BRET. HEK cells were co-transfected with equal amounts of plasmid encoding receptors coupled to RLuc8 (RL8) or YPet (Y) and the BRET ratio was measured. **Basal BRET (lower part):** Net BRET values were determined for all receptor combinations. Each pair was tested in two BRET configurations (Y/RL8 or RL8/Y). Homomeric interactions were tested by transfection cells with equal amounts of the same receptor tagged with YPet or RLuc8. **Ligand-induced BRET ratio changes (upper part)**: The effect of the ligands GIP, GLP-1, GLP-2, and glucagon on the basal BRET was assessed by subtracting the BRET value after ligand addition from the basal value. Each receptor combination was tested with both specific ligands or the two receptors; cells transfected with just one receptor type were tested with one ligand only. Decreases in the BRET ratio induced by ligand resulted in negative, increases in positive values. Each condition tested was graphed as the average +/- S.E.M. of three independent experiments. Statistical significance: ***** = $p < 0.05$; ****** = $p < 0.01$; ******* = $p < 0.001$

Figure 3: BRET donor saturation experiments. Specificity of receptor homo- and heteromerization between the GLP-1R and GIPR was tested in BRET donor saturation experiments. HEK293T cells were co-transfected with a fixed amount of

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

BRET donor plasmid and increasing amounts of acceptor plasmid. The energy transfer was tested in absence or at saturating concentrations of GLP-1 or GIP. **(A) GLP-1R/GIPR:** increasing amounts of BRET acceptor protein resulted in an increase of the BRET signal that was quasi-linear. The presence of GLP-1 increased the BRET signal at each condition tested and resulted in earlier BRET signal saturation. **(B) M4R/GLP-1R:** as a control condition, GLP-1R was co-transfected with the M4R. The curve observed is quasi-linear and no difference between the basal condition and GLP-1 treatment was observed. **(C+D) GLP-1R/GLP-1R and GIPR/GIPR:** Saturation experiments in cells transfected with just one receptor type showed the beginning of saturation in the conditions tested for both receptors. Both GLP-1 and GIP decreased the BRET signal when BRET acceptor protein was present in excess. All graphs show one representative experiment (duplicate datapoints +/- S.D) of 3 independent experiments.

Figure 4: Dynamics of the BRET signal of GLP1R/GIPR in response to GLP-1 and GIP. (A) Representation of a dynamic BRET readout of 5 min with increasing concentrations of GLP-1 added online. Traces (data point duplicates) were smoothened (average of 3 nearest neighbours, 2nd order smoothening polynominal) and buffer subtracted. (B) Representation of a dynamic BRET readout of 10 min. GLP-1 (black and dark grey) or buffer (light grey) were added online, followed by a second addition of GIP after 1 min (black) or 5 min (dark grey) or of GLP-1 (light grey) after 5 min.

Figure 5: Effects of glucagon receptor family agonists and antagonists on the heteromerization of GLP-1R/GIPR: (A) Dose response curves of were graphed by plotting the average net BRET over 10 min after a single addition of increasing concentrations of ligand. GLP-1, GLP-1(9-36) and GIP were tested for their effect

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

alone. GIP and Pro-3-GIP were tested at an $EC_{(80)}$ concentration of GLP-1 (1.3 μM). Data represent the average (+/- S.E.M.) of 3 independent experiments. (B) Dose response curves of GLP-1 were carried out in the presence of several fixed concentrations of GIP resulting in a shift of the GLP-1 curve. Representative experiment; quadruplicate datapoints (+/- S.D.). (C) Further naturally occurring glucagons-like ligands and their derivates were tested for their effect on the heteromerization of GLP-1R and GIPR. All ligands were tested in full dose response and their maximal effect at 10 μM was compared to the maximal response of GLP-1 (100%). Ligands that were tested in antagonist mode were co-added with an $EC_{(80)}$ concentration of GLP-1 (1.3 μM). $EC_{(50)}$ and $IC_{(50)}$ values were derived from full dose response curves of the ligands. Data represent that average (+/- S.E.M.) of 3 independent experiments.

Figure 6: Ligand-induced receptor internalization: The effect of the ligands on the internalization of GLP-1R and GIPR was studied in HEK cells co-transfected with GLP-1R-ECFP and GIPR-YPet. Cells were incubated with ligand (1 μM) for 20 min at room temperature, fixed, and the localization of each receptor was assessed on a fluorescent microscope.

Figure 7: Co-expression of GLP-1R and GIPR alters the GLP-1 induced, but not the GIP-induced calcium response in HEK cells. Cells were transiently transfected with a single, wild type receptor or co-transfected with the GLP-1R and a second glucagon family receptor. Changes in intracellular calcium levels were measured using the calcium-sensitive fluorescent dye Fluo4. (A) The calcium-signal was measured in response to GIP or GLP-1 in cells expressing GIPR or GLP-1R alone. The signal was normalized to the maximal response obtained by GLP-1 on the GLP-1R. (B) The GLP-1 and GIP response of the GLP-1R in presence of a second receptor

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

was tested and graphed normalized to the GLP-1 response of the GLP-1R expressed alone. Data represent the average of three to five independent experiments (+/- S.E.M).

Figure 8: Co-expression of GLP-1R and GIPR alters the GLP-1 induced recruitment of β-arrestin to the receptor. HEK cells were transiently co-transfected with GLP-1R-RLuc8 + YPet-β-arrestin2 or with GLP-1R-RLuc8 + GIPR (wild type) + YPet-β-arrestin2. The GLP1-induced recruitment of YPet-β-arrestin2 to the luciferase tagged GLP-1R resulted in an increase of the BRET ratio. The effects of dose responses of GLP-1, of GLP-1 at a fixed concentration of GIP (100 nM), of GIP at saturating levels of GLP-1 (1 μM), and of a fixed dose of GIP (100 nM) alone was compared in the absence (A) or presence (B) of the co-expressed GIPR. Representative experiment, duplicate data points (+/- S.D.).

Figure 9: Heteromerization model for GLP-1R/GIPR. (A) In the basal state the main population of GLP-1Rs and GIPRs exist mainly as mono- or homomers. GLP-1 is a high affinity ligand for GLP-1R that will activate a signaling response starting at concentrations <30 nM. (B) GLP-1 is also a low-affinity ligand for GIPR and binds at concentration >30 nM. GLP-1R and GIPR form heteromers when GLP-1 is bound to both receptors. The heteromerization with the GIPR regulates the maximal GLP-1 response of arrestin recruitment and calcium mobilization. (C) In presence of GIP, a high affinity ligand for GIPR, GLP-1 orthosterically competes with GIP for the same binding sites. With its higher affinity, GIP can displace GLP-1 from the GIPR, which results in a dissociation of the receptor heteromer and the normal arrestin recruitment profile of the GLP-1R is restored.

Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1: Calcium release of HEK cells expressing GIPR, GLP-1R or co-expressing GLP-1R with a second glucagon receptor: The $EC_{(50)}$ and Hill Slope values from independent experiments were averaged and compared for each condition. GIPR (GIP n=4; GLP-1 n=4), GLP-1R (GIP n=4; GLP-1 n=5), GLP-1R + GIPR (GIP n=4; GLP-1 n=4), GLP-1R + GlucR (GLP-1 n=3), GLP-1R + GLP-2R (GLP-1 n=3).

	GLP-1		GIP	
	$EC_{(50)}$	**Hill Slope**	**$EC_{(50)}$**	**Hill Slope**
GIPR	-	-	5.0 +/- 1.2 nM	0.59 +/- 0.27
GLP-1R	41.2 +/- 11.0 nM	1.17 +/- 0.17	-	-
GLP-1R + GIPR	**5.0 +/- 2.6 nM**	**0.50+/- 0.19**	2.35 +/- 0.5 nM	0.63 +/- 0.24
GLP-1R + GlucR	26.2 +/- 10.6 nM	1.14 +/- 0.22	-	-
GLP-1R + GLP-2R	17.0 +/- 2.3 nM	1.02 +/- 0.16	-	-

Figure 1

```
Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT---------- 29
GIP      YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ 42
GLP-1    HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG---------- 31
GLP-2    HADGSFSDEMNTILDNLAARDFINWLIQTKITD-------- 33
         :::*:* .: .  ::    ::*: **:
```

Figure 2

Figure 3

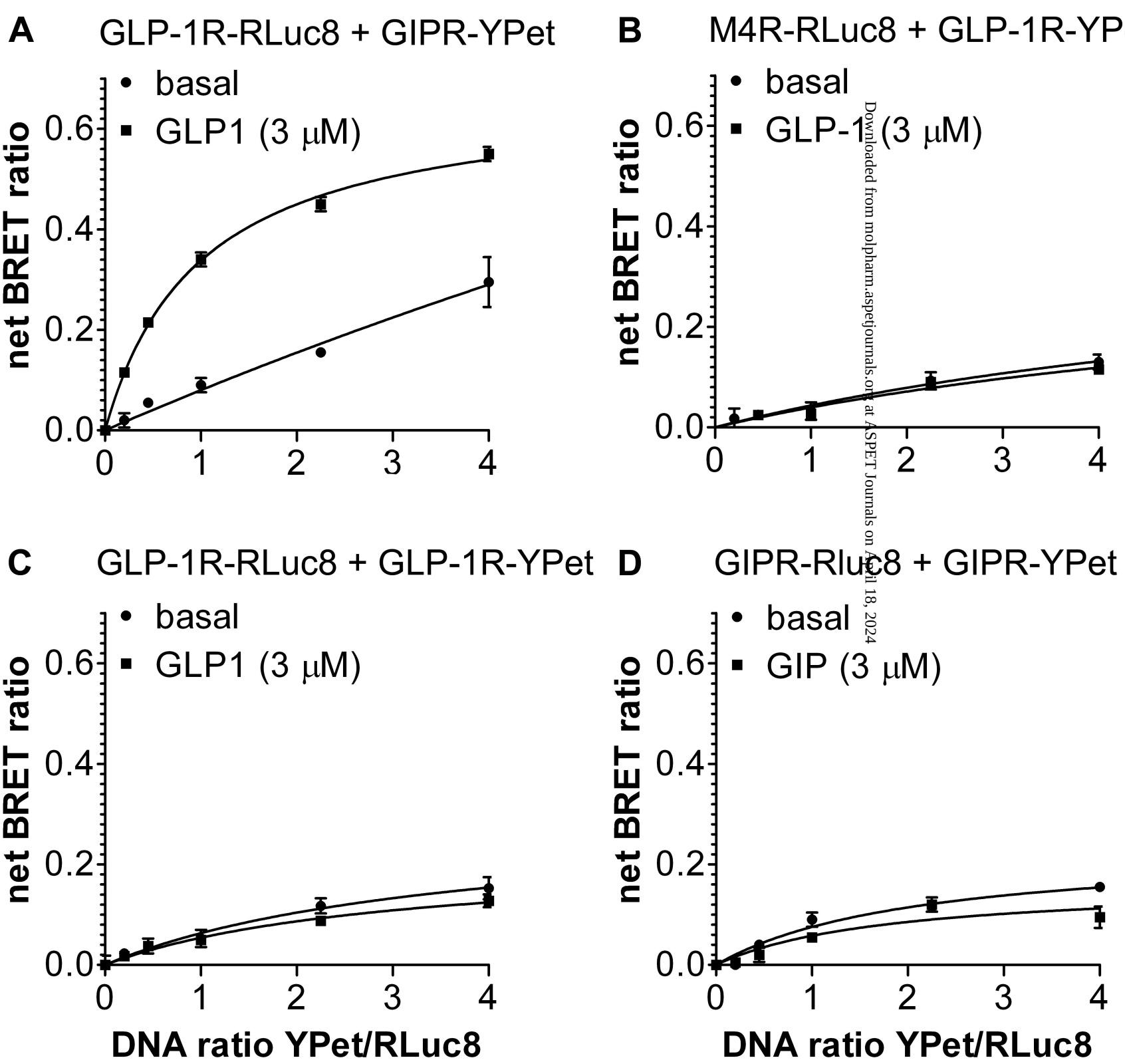


Figure 4

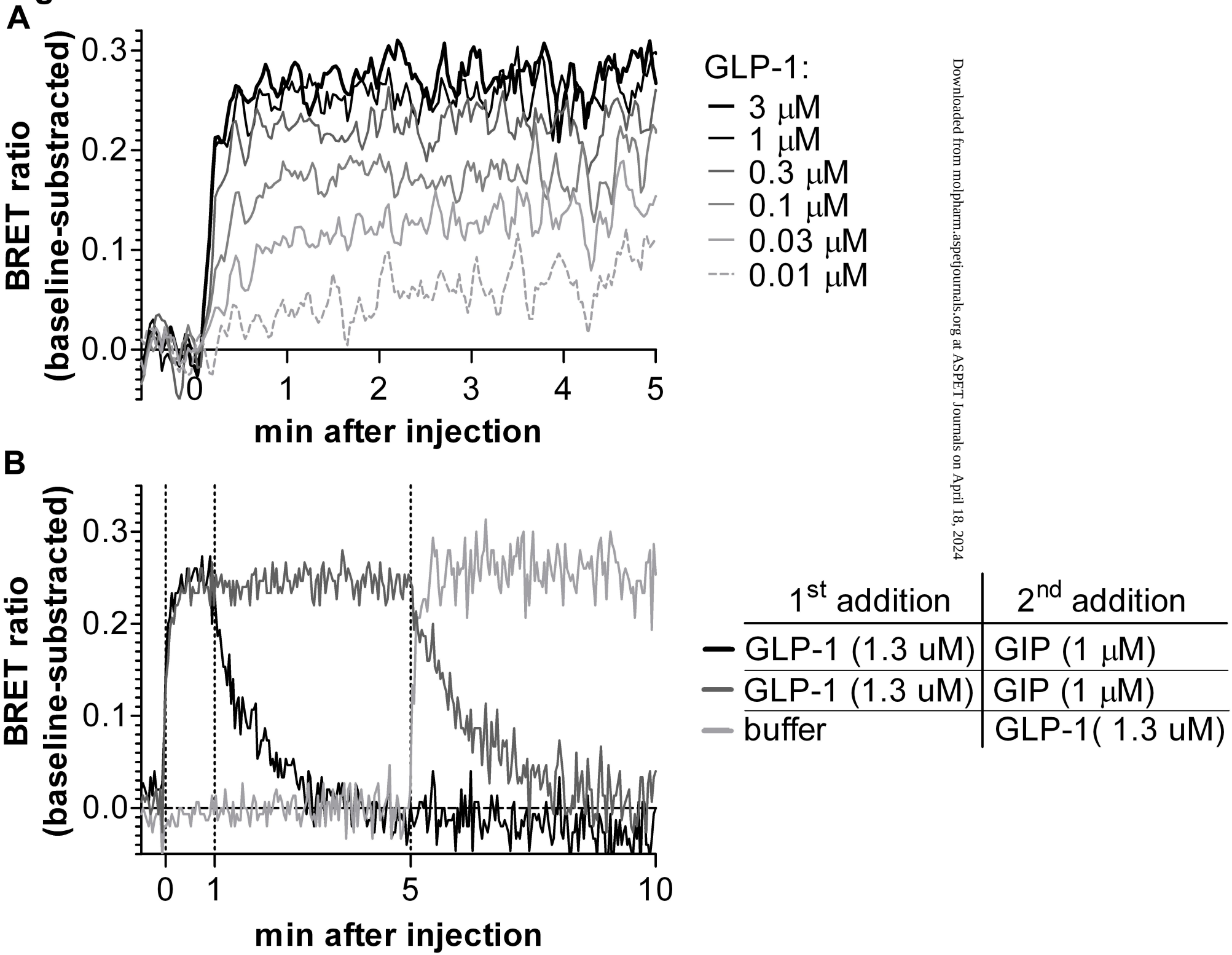
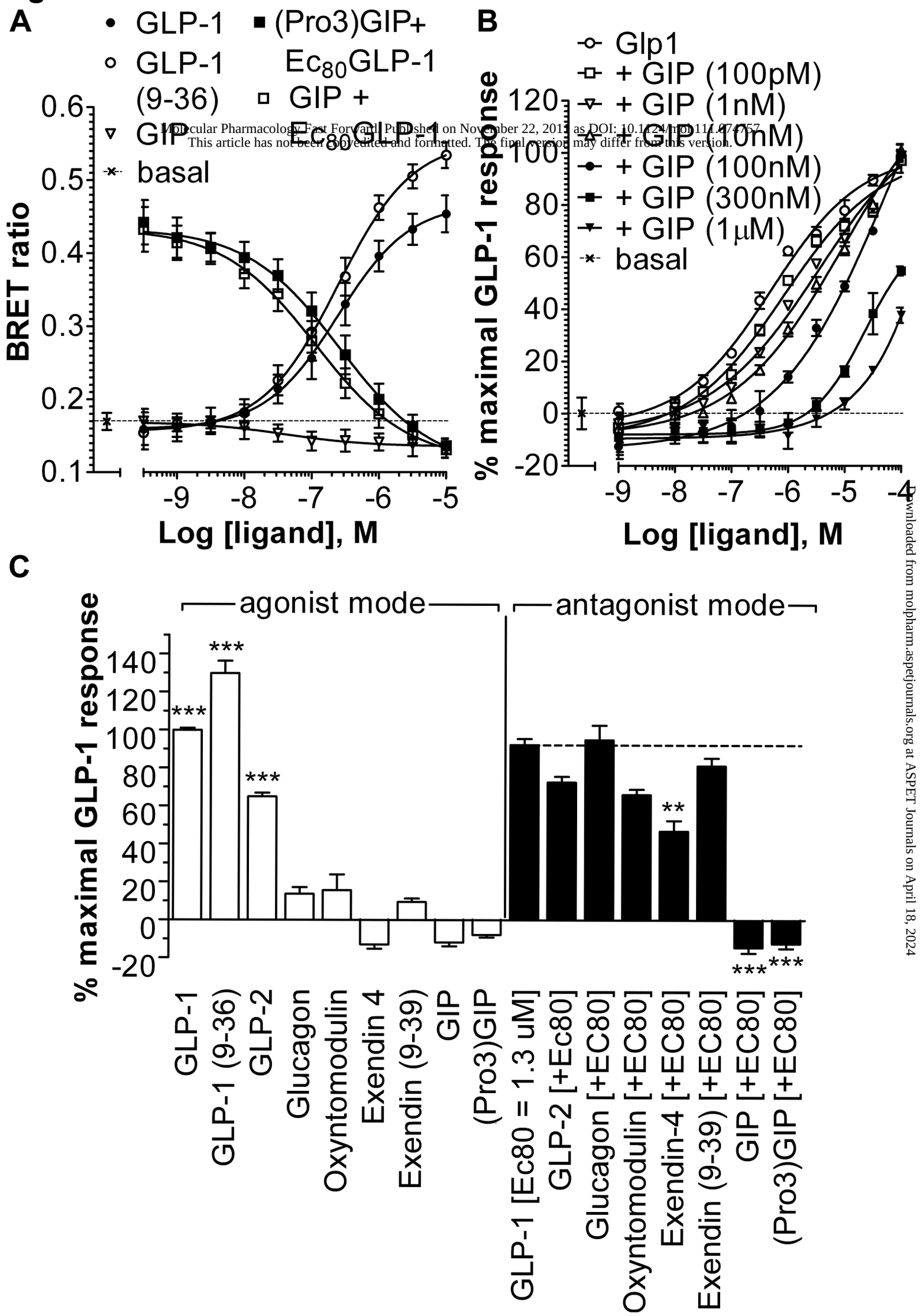


Figure 5



Molecular Pharmacology Fast Forward. Published on November 22, 2011 as DOI: 10.1124/mol.111.074757
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6

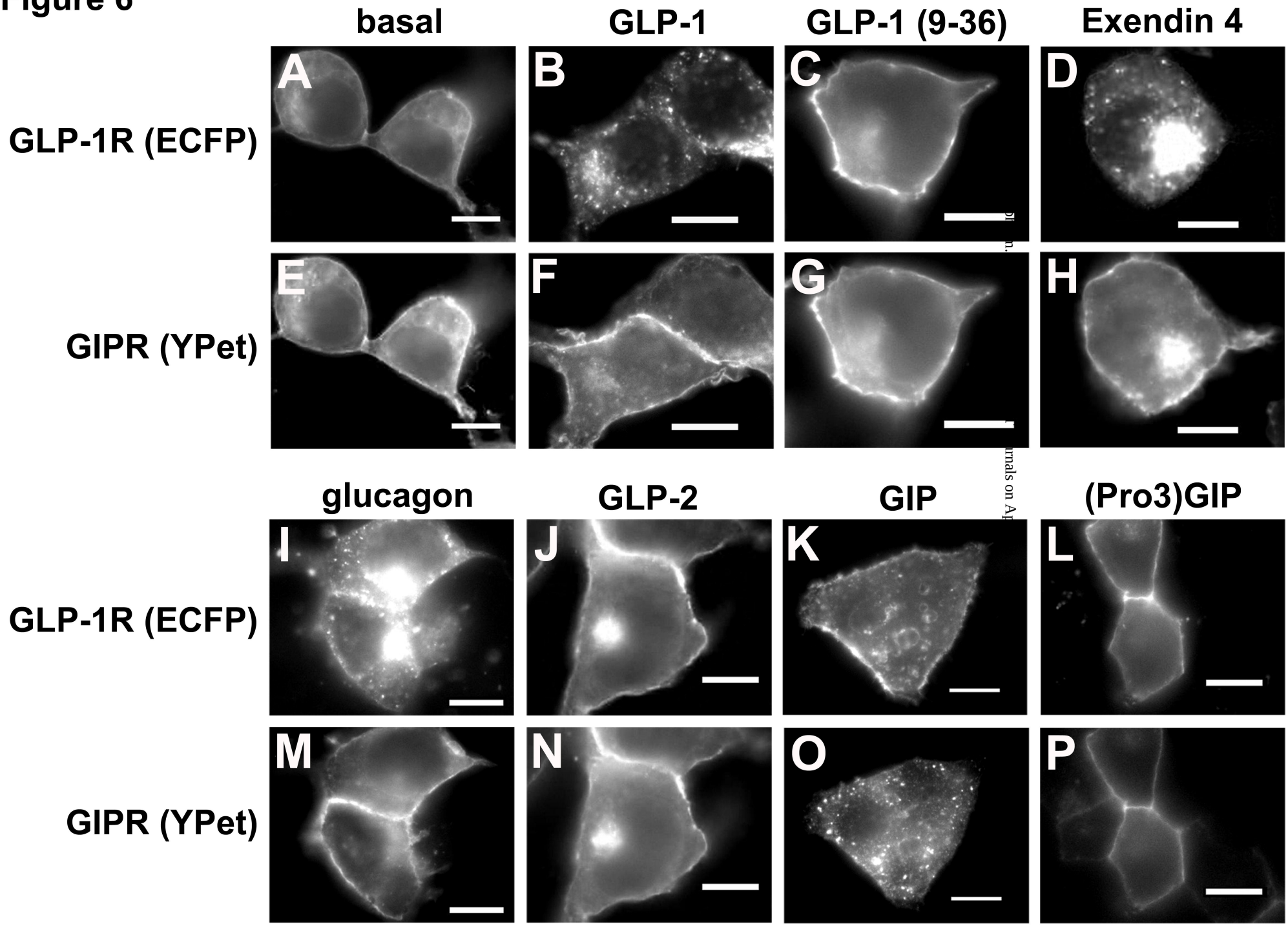

basal
GLP-1
GLP-1 (9-36)
Exendin 4
GLP-1R (ECFP)
GIPR (YPet)
A
B
C
D
E
F
G
H
glucagon
GLP-2
GIP
(Pro3)GIP
GLP-1R (ECFP)
GIPR (YPet)
I
J
K
L
M
N
O
P

Figure 7

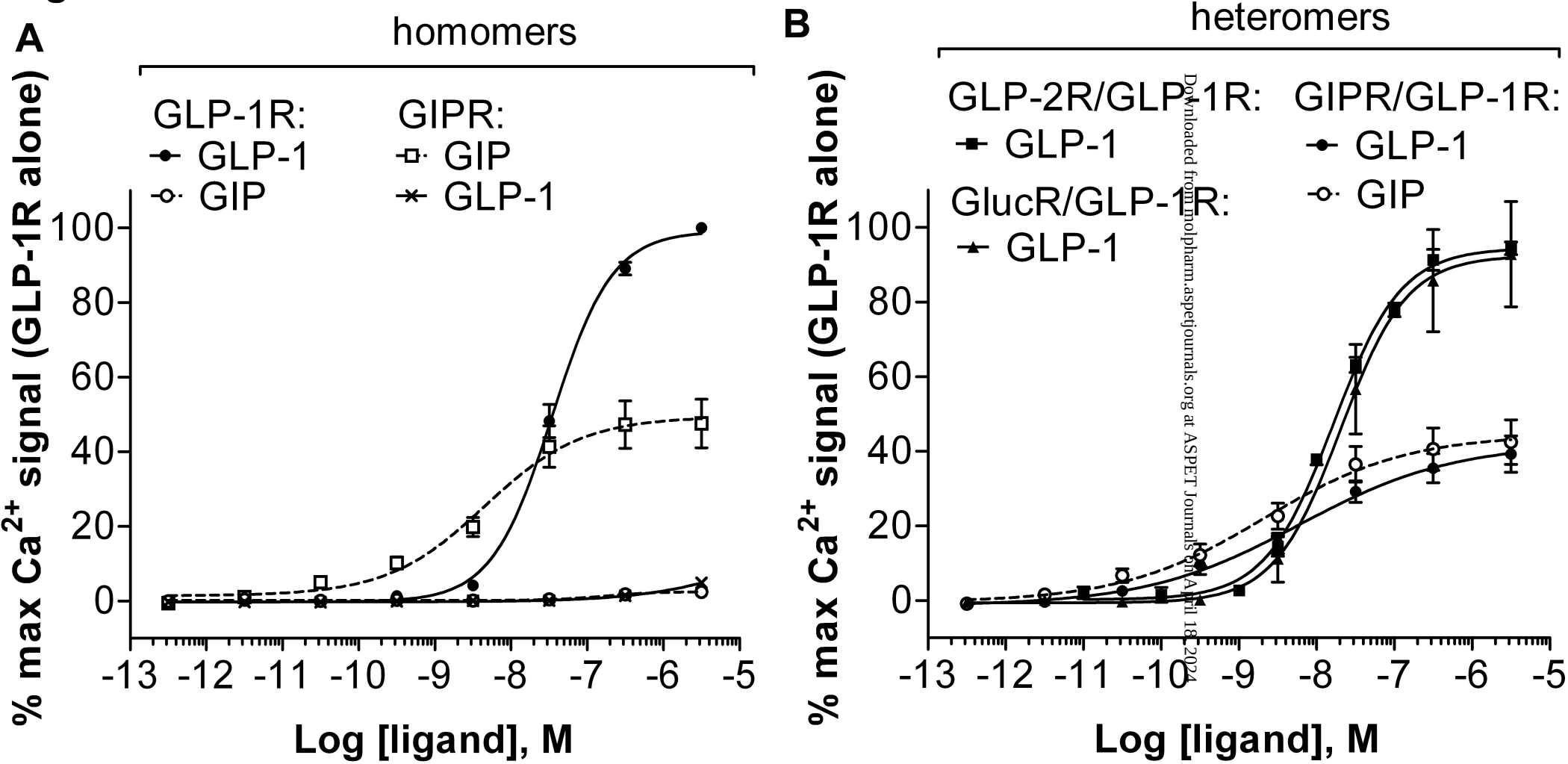


Figure 8

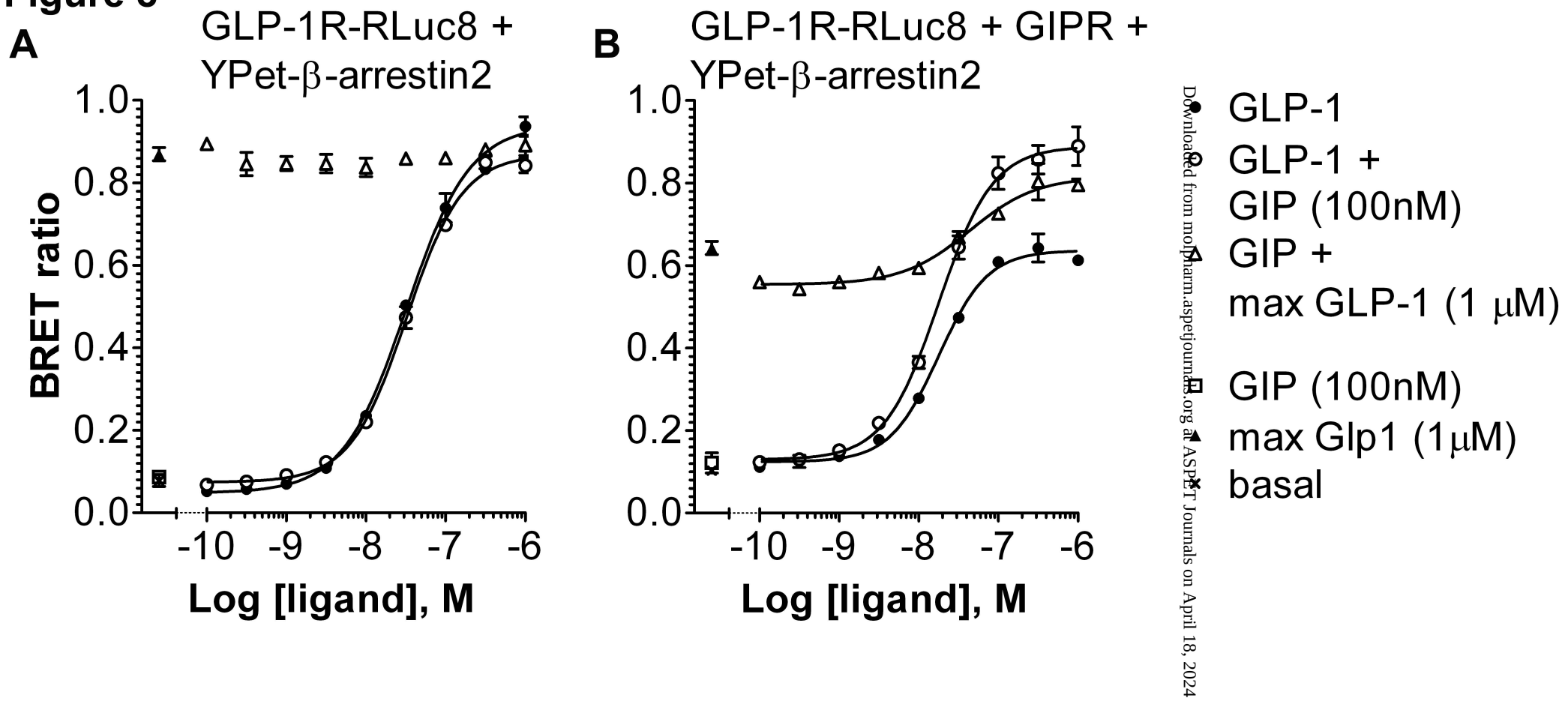


Figure 9